Genetic Studies with Herpes Simplex Virus Type 1.

by

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SUMMARY

The work presented in this thesis was concerned with the genetical analysis of Herpes simplex virus Type 1 (Glasgow strain 17). The study involved the use of two types of mutant:-

(1) Conditional lethal mutants of the temperature sensitive (ts) type which are able to grow at 31° (the permissive temperature) but not at 38° (the non-permissive temperature). (The wild type virus grows equally well at both temperatures).

(2) A plaque morphology mutant with extreme syncytial morphology (syn) derived from the non-syncytial (syn+) wild type. These mutants were isolated by Professor J. H. Subak-Sharpe.

The experiments presented in Results I were concerned with some of the general physiological properties of the wild type and mutant viruses. In growth experiments, it was found that the amount of growth exhibited by each of the mutants at the non-permissive temperature was appreciably lower than at the permissive temperature. Using heat inactivation experiments to investigate the nature of the ts defects, the results showed that none of the mutants was more or less heat stable than the wild type virus. From an analysis of the effect of the source of the virus on infectivity, it was concluded that there was a difference in infectivity depending

on whether the virus was spontaneously or artificially released from cells.

In order to determine the number of functional groups so far identified, ts mutants were studied for their ability to complement in mixed infections at the non-permissive temperature (Results II). Using two types of complementation assay, the nine ts mutants were assigned to eight cistrons. Making use of the two types of test substantiated the assignments.

Two-factor crosses involving different pairs of ts mutants were used to examine the possibility that a recombination mechanism existed for HSV:1 (Results III). The proportion of progeny plaques which plated at 38° (the property of ts+ virus) was increased among the progeny of mixed infections relative to the control single parent infections. This result indicated that recombination did occur and was substantiated by showing in progeny tests that the putative ts+ recombinants were in fact true recombinants. Non-syncytial revertants isolated from the original ts syn mutants were used in reciprocal three-factor crosses of the type; tsXsyn x tsYsyn+ and tsXsyn+ x tsYsyn. With recombination data from these crosses a linkage map has been constructed. The provisional map which locates nine cistrons is linear, gives reasonably additive map distances and spans about 25 map units.

The nature of plaques with mixed syn/syn+ morphology which were a regular occurrence in the progeny from syn x syn+ crosses

was investigated using physical and genetical techniques (Results IV). The results so far obtained, suggest that the mixed plaques are produced from single virus particles which are genetically heterozygous and not from some association of homozygous particles.

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INTRODUCTION

I. Herpes Viruses.

A. Discovery.

The word Herpes, derived from the Greek word ἕρπειν meaning to creep has been used in medicine for more than twenty five centuries. In the Hippocratic Corpus the term was applied to spreading cutaneous lesions of varied etiology (Beswick, 1962). An account of Herpes labialis appeared in the seventeenth century, followed by cattarhalis, pro genitalis, faecalis and simplex in the eighteenth and nineteenth centuries.

The first to show that Herpes febrilis was infectious was Vidal in 1873. However it was not until 1919 that any interest was shown in this finding, after Lowenstein published evidence that Herpes keratitis and labialis yielded a virus that would produce characteristic lesions on the cornea of a rabbit. In 1938 Doerr questioned the role of Herpes simplex as an infectious agent as it did not appear to be transmitted from one individual to another but arose endogenously within the individual after non specific stimuli. Contrary to all the rules of virus-host interactions was the finding by Andrewes and Carmichael (1930), that most normal adults in a population possess neutralising antibody against Herpes simplex virus in the blood and that recurrent Herpes infections develop only in those with neutralising antibody. In 1938, Dodd elucidated the role of Herpes simplex virus

as a disease producing agent by isolating the virus repeatedly from the mouths of infants with an acute form of vesicular stomatitis. The role was confirmed by Burnett and Williams (1939) who demonstrated that:- (1) Infants developed neutralising antibody during the periods of convalescence from vesicular stomatitis and that (2) Herpes simplex infections appear to persist for life and that virus can be isolated from individuals with recurrent infections. It soon became evident that primary infections by Herpes simplex virus are common in man and after recovery the virus appears to enter into a latent state. Only after the discovery of the causative agent did it become clear that Herpes genitalis, faecalis and febrilis were all different clinical manifestations of the one disease, Herpes simplex.

B. Catalogue of viruses belonging to the Herpes group.

Herpes viruses are defined as large enveloped virions with a well defined icosahedral capsid consisting of 162 capsomeres arranged around a DNA core. The viruses usually included in the group are Herpes simplex, B virus, marmoset virus, pseudorabies virus, equine herpes, varicella zoster virus, cytomegalo virus, a number of bovine, canine, avian, rabbit and feline herpes viruses. Among the most interesting aspirants for inclusion into the group are the viruses associated with Burkitt's lymphoma, Marek's disease of fowls and possibly the virus associated with Lucke's adenocarcinoma of frogs.

C. Structure.

Information concerning the structure of Herpes viruses has been basically derived from three sources.

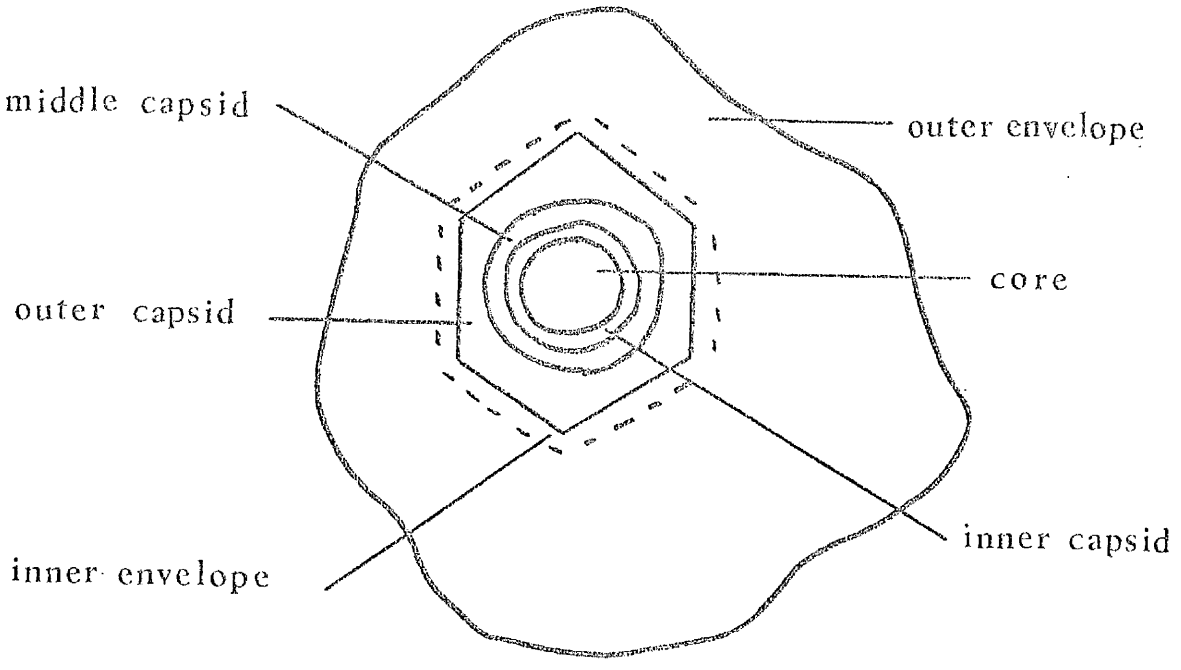
(1) Thin sections of infected cells observed in the electron microscope.

(2) Negatively stained preparations of virus observed in the electron microscope.

(3) Chemical and biological studies of isolated components.

In this study we are primarily interested in Herpes simplex virus and therefore the following information is confined to this member of the Herpes group. The virus consists of a core, containing double stranded DNA of molecular weight 105×10^6 daltons (Wilkie, 1972). The genome size and redundancy has been studied by renaturation kinetics (Frenkel and Roizman, 1971). The DNA was sheared to uniform fragments, denatured by heating and allowed to reassort. The reactivation reaction was shown to follow second order kinetics with a single rate constant, indicating that at least 95% of the genome is unique and that repetitive sequences if present were not detectable. The kinetic complexity of the genome was determined by the DNA renaturation kinetics to be $95 \pm 1 \times 10^6$ daltons. Since this was in excellent agreement with the molecular weight of viral DNA obtained by velocity sedimentation studies, it was concluded that virions contain only one species of double stranded DNA molecules. Evidence of this kind on the nature of HSV, DNA is important from the point of view of genetic studies.

The core and surrounding capsid combine to form a nucleocapsid which is in turn surrounded by an envelope. A number of structures have been observed which are thought to make up the enveloped nucleocapsid. These are (a) a core 25-30 nm in diameter, (b) an inner capsid 10 nm thick, (c) a middle capsid 15 nm thick, (d) an outer capsid 12-15 nm thick and consisting of 162 capsomeres, (e) an inner envelope 10 nm thick and (f) an outer envelope approximately 20 nm thick. A diagram of the structure of HSV is shown below.



The presence of six of these components has been discerned and confirmed by more than one source (Morgan et al., 1953, 1954, 1958, 1959, 1960; Epstein, 1962a, 1962b; Spring and Roizman, 1968). The most extensive studies of the viral capsid were those carried out by Wildy et al., (1960). Their data showed that the nucleocapsid of the virus contained 162 capsomeres arranged in 5, 3, 2 axial symmetry to form an icosahedron. The experiments, carried out using phosphotungstate acid staining, showed that the capsomeres were hexagonal in shape. The existence of a middle capsid was first reported by Wildy, who observed a particle 77.5 nm in diameter which was calculated to be able to be derived from a complete particle (105 nm) by stripping a layer of capsomeres 12.5 nm thick.

Spring et al. (1968) found a particle similar in size to that observed by Wildy in preparations of virus which had been centrifuged through CsCl. The core of the capsid would appear to be surrounded closely by a beaded structure which has been called the inner capsid. Bearing in mind the reservations which have to be taken into account when analysing electron microscope observations, it would appear from the reports that the structure of the nucleocapsid has been fairly thoroughly elucidated and confirmed.

D. Envelopment and the role of the envelope.

One of the most controversial aspects of the structure of the herpes virion is the source of the envelope and the role of the

envelope in the infectious process. The envelope has been estimated to be about 20 nm thick but can vary considerably in size and shape from being quite tightly adhering to the capsid to being large, floppy and amorphous, (Spring et al., 1968). Although numerous papers have dealt with the source of the envelope in various cell lines, no unambiguous conclusions have been reached. The main problem seems to be in the analysis of electron micrographs of thin sections of infected cells and whether it is valid to compound the static pictures obtained into an overall sequence of events during the process of envelopment of the nucleocapsid. In spite of these problems of interpretation, general agreement has been reached on certain points:- (1) The site of envelopment is in general the nuclear membrane. Partially enveloped particles are most frequently seen in apposition to the inner lamellae of the nuclear membrane and enveloped particles are observed between the inner and outer lamellae (Morgan et al., 1959; Watson et al., 1964). (2) It would appear that envelopment may also occur in the cytoplasm. Frequently partially enveloped particles are observed in the cytoplasm in apposition to undefined cytoplasmic membranes (Epstein, 1962a). (3) It is thought that the enveloped particles in the cytoplasm are transferred to the outside of the cell in vacuoles by a process akin to reverse pinocytosis. However tubules have been seen originating from the outer lamellae of infected cells and opening into the extracellular fluid (Schwartz and Roizman, 1969). These have been seen to contain enveloped

virions. It would appear therefore that there may be two forms of egress of the enveloped virion into the extracellular fluid.

It has been suggested that, as well as the structure which is generally referred to as the envelope, there is another structure which has been called the inner envelope and this is thought to fill the space between the capsid and the outer envelope. It is postulated that only nucleocapsids which are covered by this inner envelope acquite an affinity for the nuclear membrane and so obtain an outer envelope. The discovery of enveloped virions (Wildy et al., 1960), raised the question whether either or both are infectious. Different preparations of virus vary greatly in the relative proportions of enveloped and naked particles. Holmes and Watson (1961) corroborated by Siegert and Falke (1966) reported that enveloped virions were more readily adsorbed to cells than naked ones but they indicated that this may only reflect a size difference. Subsequently (Watson et al., 1964) reported that in some preparations the number of plaque forming units exceeded the number of enveloped particles. This led to the conclusion that both particles were probably infectious but not with the same efficiency. This conclusion was challenged by Smith (1964) who reported separation of enveloped and non-enveloped particles by iso pycnic centrifugation in CsCl. However CsCl was found to be deleterious by causing disaggregation of the virion (Spring and Roizman 1967; Spring et al., 1968).

Although it is without doubt that enveloped particles are infectious, the question as to whether or not naked particles are so requires the absolute separation of the two types of particle. Numerous attempts have been made to separate the particles and some have had a limited amount of success. There have been reports of separation using potassium tartrate gradients (Norcross et al., 1963) and sucrose gradients (Levitt and Becker, 1967; Olshevsky and Becker, 1970; Stein et al. 1970; Aurelian and Wagner, 1966). However even although separation is attained at the level of 99% of one type of particle in a band, the 1% of the other type does not permit an unbiased answer to the question. The envelope can easily be removed by using e. g. detergents but then the question is whether the lack of infectivity is due to the fact that the particle does not have an envelope, or to some other deleterious effect of the detergent. The question could be answered using ts mutants if complete separation could be achieved. By mixedly infecting cells with enveloped particles of one ts mutant and naked particles of another complementing ts mutant and determining whether complementation or recombination was taking place it could be ascertained whether or not naked particles were, even if not fully infectious at least still biologically active. However for the present a complete separation has not been achieved. The real problem lies in the fact that there is a wide spectrum of particles from completely naked to completely enveloped. Recently (Schaeffer et al., 1971)

have isolated a ts mutant of HSV Type 1 which is lacking in its envelope protein but is still infectious at the permissive temperature.

E. The infectious process.

(1) Adsorption.

The process of adsorption of HSV to cells has been studied using the electron microscope. The rate of adsorption is known to be dependent on the volume of virus inoculum, the presence of cations, the metabolic state of the cells but not on the temperature of incubation within the limits of 0-37°, during the process. Holmes and Watson (1961) found that attachment and penetration of virus particles were dissociated and that enveloped particles adsorbed more readily than naked ones. However, Hochberg and Becker (1968) found that both enveloped and naked HSV particles were equally adsorbed to BSC 1 cells and concluded that both types of particle were infectious. They found that adsorption to the cell surface started immediately on the addition of virus and that the number of particles on the cell surface gradually increased until 3 hrs after infection. Adsorption was found not to be affected by the addition of inhibitors of protein and nucleic acid synthesis. Using heparin they found that the first stage in the attachment of the virus to the cell surface was electrostatic in nature. As yet nothing is known of the nature of cell receptors and cells naturally lacking receptors for HSV have not been described.

(2) Penetration.

The process of penetration has also been studied using the electron microscope. The process is known to be temperature dependent. Holmes and Watson (1961) working with infected BHK21/C13 cells concluded that the virus was taken in by a process of pinocytosis. They pointed out the limitations of this type of investigation in that it is never certain whether the actual infecting particles may be undergoing processes rather different from that undergone by the majority of the observed particles. Although they felt certain that the infecting particles enter by pinocytosis they did not exclude the possibility of undetected particles entering by another route. Morgan et al., (1968) working with HSV-infected HeLa cells, listed five steps in the process of initiation of infection. These are (1) attachment, (11) digestion of the viral envelope, (111) digestion of the cell membrane, (1v) passage of the nucleocapsid directly into the cytoplasm and (v) digestion of the capsid with release of the core. Morgan suggested that although pinocytosis cannot be ruled out as the process by which HSV initiates infection, three observations seemed contrary to this concept. First pinocytosis cannot explain the formation of polykaryocytes which result from cell fusion. Secondly in their observations it was far more common to encounter virus in the process of digestion at the cell surface than to find it within vacuoles. Thirdly, whereas capsids and cores were repeatedly found in the cytoplasm, stages in their

release from vacuoles were not observed. Morgan's observations showed that the first stage of entry appeared to be attachment of the viral envelope to the cell surface, followed by the disintegration of the viral envelope adjacent to the cell surface. The temperature dependence of this reaction suggested that it was due to enzymic digestion. The remaining viral envelope was then shown to fuse with the cell wall and the wall of the cell subsequently disintegrated. It was suggested that an enzyme capable of digesting the cell wall is activated by the virus at the site of attachment. The existence if a third enzyme capable of attacking the capsid within the cytoplasm was also postulated. It would appear therefore that there may be two alternative mechanisms involved in the process of penetration. The fact that Morgan et al. are the only workers who have observed the events described above,could be explained by the fact that the events occur quickly and could therefore be easily missed or that they have been seen but not recognised. Further light may be thrown on the subject when the enzymes involved in the process of entry have been isolated and characterised.

(3) Reproductive cycle.

The duration of the reproductive cycle is known to be affected by the temperature of incubation, the type of cell being infected, the physiological state of the cells, the virus strain, the multiplicity of infection and prior infection of the cells. The

yield from infected cells increases from the end of the eclipse phase to the end of the cycle. The length of the growth cycle varies from about 12-24 hours and the burst size is on average 100 infectious particles/cell depending on the factors stated above.

F. Plaque formation.

Plaque formation is based on the principle that an infectious virus which comes into contact with a susceptible cell will multiply within the cell and destroy it. The newly formed virus particles will then be released and spread to the other cells in the culture. If this process is confined to the neighbouring cells, a recognisable focus of destroyed cells will be formed for every infectious viral particle which has infected a cell of the monolayer culture. With HSV, plaques are also formed without the release of virus from the cells. These are syncytial plaques and are formed by the fusion of an infected cell with the surrounding non-infected cells to form a giant multinucleate fused cell.

Using the finding of Andrewes (1930), that Herpes will multiply even in the presence of immune serum, Black and Melnick (1955) showed that plaques of Herpes B virus will form in monolayers of monkey kidney cells incubated with specific antiserum. This has since been shown to be true with other members of the Herpes group. The antiserum prevents virus released into the medium from infecting

cells at random but does not prevent the passage of infectious virus from cells to neighbouring cells, thus permitting recognisable plaques to be formed. The size of the plaques formed depends on the virus strain and the cell type. Cells may also be infected in suspension and then plated in petri dishes. The non-infected cells grow to form monolayers in which the infected cells form foci of infection and secondary plaque formation is prevented by the addition of antiserum to the overlay medium (Russell, 1962).

G. Macromolecular synthesis during the reproductive cycle.

(1) DNA.

Recent studies on the host cell and the viral DNA synthesis in synchronous cultures of KB cells infected at different stages of the cell cycle with HSV showed that initiation of DNA synthesis is independent of S, G1 and G2 phases of the mitotic cycle in the host cell. HSV DNA synthesis was initiated 2/3 hours after infection. The rate of synthesis was shown to increase rapidly reaching a maximum 4 hours after infection and decreasing to 50% of the maximum by 8 hours. It was concluded that HSV can inhibit both the on going synthesis of host DNA as well as the initiation of the S phase (Cohen et al., 1971).

For successful completion of the infectious process HSV requires the infected cell to contain the enzymes needed for both de novo DNA synthesis and for DNA synthesis derived from breakdown

products. Perera (1970), showed that in mammalian cells infected with HSV, viral DNA can be formed from apparent breakdown products of cellular DNA and that in certain circumstances the infected cell may derive much of its entire pool of deoxyribonucleotides from this source. However Levitt and Becker (1967) produced evidence that viral DNA precursors may be produced by continuing de novo synthesis in the infected cell.

(2) RNA.

Evidence has been presented that transcription of viral DNA is required for virus multiplication; that viral RNA is synthesised and that new species of polyribosomes appear in the cytoplasm of infected cells. Annealable RNA i. e. virus specified RNA reaches peak levels 6-7 hrs after infection and declines slowly thereafter (Flanagan 1967). From 6-14 hrs the sedimentation patterns and the size of the RNA molecules annealable to DNA remains constant.

Chromatographic and hybridisation studies on the 4S RNA from HSV infected cells suggested the presence of virus specified arginyl tRNAs (Subak-Sharpe and Hay, 1965; Subak-Sharpe et al., 1966). However, further studies using improved techniques have shown that no detectable HSV specified tRNAs are present in cells 7-9 hrs post infection (Bell et al., 1971).

(3) Protein synthesis.

All protein synthesis in Herpes infected cells has been shown to occur in the cytoplasm (Sydiskis and Roizman, 1966; Fujiwara and Kaplan, 1967). It has been shown that within a short time after infection, host polyribosomes begin to disaggregate and the spectrum of proteins and glycoproteins made in the infected cell changes rapidly (Sydiskis and Roizman, 1967). The evidence that these new polyribosomes are viral is based on the finding that the RNA associated with them, hybridises to viral DNA even in the presence of large amounts of host RNA (Wagner and Roizman, 1969 a, b). The synthesis of protein as measured from the rate of incorporation of amino acids and the amount of polyribosomes in the cytoplasm has been shown to reach peak levels 4-6 hrs post infection and thereafter slowly decline. However, it has been shown that there is substantial protein synthesis as late as 12 hrs post infection and the analysis of proteins made at different times after infection, suggested that the synthesis of structural proteins may be asynchronous (Spear and Roizman, 1968). Transport of viral proteins from the cytoplasm to the nucleus has been found to be relatively slow (Olshevsky et al., 1967; Spear and Roizman, 1968; Ben-Porat et al., 1969), and there is no evidence that transport may be selective (Spear and Roizman, 1970).

The level of activity of certain enzymes rise as a result

of infection with HSV. These include DNA polymerase, DNase (Keir and Gold, 1963), dCMP deaminase (McGeoch and Keir, 1968), thymidine kinase (Kit and Dubbs, 1963a) and dTMP kinase (Newton, 1964), dCyd kinase (Hay et al., 1971). In general in cells infected with HSV, deoxypyrimidine kinases are induced and deoxy purine kinases are not (Perera, 1970).

At the moment there is no reliable method for differentiating between structural and non-structural proteins. Twenty four bands of protein in HSV infected HEp 2 cells have been identified electrophoretically (Spear and Roizman, 1972). The coding potential of HSV is about 100 averaged sized proteins, therefore about 75% of the coding potential of the virus is unaccounted for at present. Provided the proteins are essential for virus production, they should be able to be identified using genetic techniques.

H. Conclusion.

Although the first experimental transmission of Herpes viruses to a heterologous host were performed more than half a century ago and that since then they have been the subject of study of many thousands of projects, relatively little information of a comprehensive nature is known about them compared to newer viruses with a much less illustrious ancestry. This is probably due to two reasons. First, the molecular biology of animal viruses evolved from studies

on pathogenesis and the prevention of the disease they cause. While Herpes virus infections of man may be severe, they are rarely crippling, seldom lethal and they never cause epidemics of great military or economic importance. Thus it came about that the methodology for the studies of Herpes viruses largely owes its existence to the pressing needs of research on other viruses. The second and perhaps more important reason for the lag in studies of Herpes viruses is that they are among the most complex viruses infecting animal cells.

One objective of the study of Herpes viruses is the complete description of the structure, functions, amount and time of synthesis of all the products specified by the virus in the infected cells. HSV DNA carries information sufficient to specify the sequence of 140,000 amino acids. At present the number of products of HSV infection and their function are uncertain. With the isolation of temperature sensitive mutants of the virus, it is hoped that Herpes specified products will be able to be identified and further light will be thrown on the processes involved in the synthesis of these products. However it seems unlikely, that with a virus with so large an information content, that the entire content will ever be determined.

The experimental work presented in this thesis describes the start of the genetic analysis of HSV Type 1 using principally temperature sensitive (ts) conditional lethal virus mutants. The ts mutants have been analysed with respect to their ability to complement and recombine and some indications of a possible intermediate in the recombination process have been obtained. Since the genetic study of animal viruses is relatively undeveloped and latterly has owed much of its impetus to studies with bacteriophages, the following section which discusses genetic analysis is based largely on phage studies.

II. Genetic Analysis.

The genetic material of a virus has two functions, (1) to replicate itself and (2) to express its information. Genetic analysis is an attempt to describe the structure and function of the genetic material of an organism by means of an analysis of its behaviour in a mutant form. In terms of the viral genome, genetic analysis may be thought of as the establishing of a total genetic map i. e. a diagram of the genome containing all the genes, their number, sequence, extent, origins and terminations in relation to the identity, structure, function and interactions of their gene products.

A. Discovery of conditional lethal mutants of viruses.

An era of virus genetics began in the early 1960's with the discovery of conditional lethal mutants of the phages lambda and T4

of Escherichia coli. The term 'conditional lethal mutant' had first been used to describe mutants of Drosophila which were either lethal under one set of growth conditions but which grew relatively normally under different growth conditions (Hocham, 1951). Until the beginning of the decade viral geneticists had used as genetic markers mutant strains which were recognised primarily on the basis of altered plaque morphology or resistance to various agents. In the late 1950's Campbell had described host defective mutants of the phage lambda (Campbell and Balbinder, 1958) but it was not however until 1961 when he isolated temperature sensitive mutants of lambda (Campbell, 1961), followed by the isolation of ts mutants of T4 that the full potential of this class of mutants started to be exploited, (Edgar and Lielausis, 1964). The discovery of the ts mutants of T4 was paralleled by the isolation of amber mutants of the same phage (Epstein et al., 1963). The amber mutants were unable to grow on E. coli strain B, the normal host of the T4 amber + wild type, but they were able to grow on other E. coli strains. Both these types of general conditional lethal mutants were shown to be easily obtained and to occur in many genes. Unfortunately suppressible mutants of the amber type have so far been unavailable in animal virus systems. The ts and amber mutants in T4 were found to be distributed over the entire length of the genome, many being located in hitherto unrecognised genes. Thus the geneticists were provided with many new markers and a simple method for detecting them. Also since the new mutants were located in genes whose expression was by definition necessary for replication, the

biochemists could ask which genes were responsible for essential phage-induced proteins. Hence the discovery of conditional lethal mutants of T4 has led to the most comprehensive and detailed genetical and biochemical analysis of any virus (Epstein et al., 1963; Edgar and Wood, 1966).

B. Nature of the temperature-sensitive defect.

Temperature-sensitive mutants have a more restricted temperature range for growth than the wild type. Usually but not invariably they will replicate at the low end of the temperature range but not at the higher. The selected temperature at which replication is prevented is called the non-permissive temperature and the selected temperature at which replication occurs the permissive temperature. Temperature sensitive mutants are missense mutations which result from alterations in the nucleotide sequence of wild type virus such that the protein product of the ts mutant gene is unable to assume its correct functional configuration at the restrictive temperature. The proof that ts mutants are missense has been provided by work with Tobacco Mosaic virus, in which changes in the amino acid sequence of the viral polypeptide has been determined for several ts mutants (Wittman and Wittman-Liebold, 1966). Usually the complete polypeptide specified by the mutated gene is synthesised but its function is defective at the restrictive but normal or nearly so at the permissive temperature. If the defective protein is a structural component,

viruses produced at the permissive temperature may be less heat stable than the wild type virions. If the defective protein is not structural, the virions have the same heat stability as the wild type. Likewise viral enzymes synthesised by ts mutants under permissive conditions may themselves be less heat stable than the wild type.

C. Advantages of conditional lethal mutants.

Since the time of the first isolation of conditional lethal mutants, they have become increasingly important tools in genetic analysis. The reasons for this are fivefold:- (1) They are easy to select as it is unnecessary to know which function is altered. (2) The mutations affect a large proportion of the viral genes. Thus a set of ts mutants may be expected to include some with altered essential enzymes, some with altered virion proteins and some with changed regulatory functions. (3) The mutants can be used to define the physiological nature of the genetic blocks by finding out for each mutant the stage at which viral development is arrested under restrictive conditions by carrying out temperature shift experiments. (4) Mixed infections of cells under restrictive conditions by two different conditional lethal mutants, each of which cannot multiply on its own, permits viral growth by complementation if the mutants are in different cistrons. In this way mutants can be arranged in different groups according to their complementation properties. In theory and in practice this is true for all phages but for some animal viruses complementation is very

inefficient. (5) The mutants allow genetic mapping by recombination tests between different mutants.

D. Complementation tests.

Having isolated a series of conditional lethal mutants, a first step in their genetic analysis is the assignment of the mutants into complementation groups (also referred to as cistrons or genes). This is done by complementation tests. Two mutants are used to infect a cell under restrictive conditions. If both mutations are located within the same gene, then both will produce the same faulty protein product. The mixedly infected cell will be deficient for an essential polypeptide and so will fail to produce progeny virus. If the mutations affect different genes, the defective protein produced by one parent will be supplied in functional form by the other parent and vice versa, the infected cell will therefore contain a complete complement of functional polypeptides and will produce progeny virus. This co-operative phenomenon is referred to as complementation. Those mutants which complement define different genes and those which do not, represent lesions affecting the same gene (except for rare intragenic complementation). Therefore given a number of ts mutants, each mutant can be assigned to a complementation group by carrying out a number of infections of this type.

The most elegant complementation tests were those carried out by Benzer on the r11 mutants of phage T4. In 1955 Benzer discovered

that the r11 mutants of T4 in addition to their typical r morphology when assayed on E. coli strain B, possessed another phenotype i. e. they could grow on E. coli K strains lysogenic for the phage lambda. On K strains the r+ wild type phage, as well as the r mutants not belonging to the r11 class could grow perfectly well. The r11 mutants were found to adsorb to K strain bacteria but they did not produce infectious phage progeny. One of the questions Benzer wished to answer was whether the phenotype of the r11 mutants of his collection was attributable to genetic lesions in more than a single functional unit. He realised that the failure of different r11 mutants to grow on the non-permissive K strain need not necessarily reflect the same functional defect in their hereditary material. To examine whether two different r11 mutants belong to the same functional unit, Benzer adapted the complementation test previously developed with higher organisms. The test was carried out by mixedly infecting K cells with two r11 mutants and examining in a spot test for the products of the yield. On the basis of this test Benzer found that the r11 point mutants fell into two complementation groups. The essential feature of the complementation test was the nature of the progeny virus. They were all found to be of the parental type and were not recombinant particles. This showed that the production of progeny was by a process of complementation and not recombination. All members of one group complemented the other in the production of infectious progeny but

did not complement any member of their own group. It was also found by mapping studies that the two groups could be assigned definite separate positions on the genetic map. The two mutant groups thus signalled the existence of two functional units within the r11 region of the phage genome; each unit presumably governing the synthesis of a specific polypeptide necessary for growth in strain K bacteria.

Benzer's work provided the basis for the later classification of a large number of ts and amber mutants of T4 into individual genes (Edgar and Epstein, 1965). In this way by 1965 some 60 genes were identified. It was not all plain sailing however, as the extension of Benzer's complementation test to other parts of the genome showed. Although the gene assignments of all the amber mutants were unambiguous, Edgar and Epstein encountered some ts mutants that, on the basis of their position on the genetic map and the results of cis-trans tests with closely linked amber mutants clearly belonged to the same gene yet gave good complementation in the cis-trans test with each other. This was the discovery in viruses of intragenic complementation and it was soon encountered in attempts to define the units of genetic function in bacteria and in other organisms (Edgar et al., 1964; Bernstein et al., 1965). It is thought that intragenic complementation can only occur when the gene product is larger than a monomer and is composed of polypeptide sub-units, where defects in the sub-units can interfere with the aggregation of the units or the configuration of the resulting

protein, so that its function is impaired or absent. When, in complementation tests, sub-units altered in different ways are synthesised in the same cell, some pairs having non-overlapping defects may be able to compensate one another's defects and so form a hybrid structure which is stable and possesses some activity but which is nevertheless different from wild type. Other pairs, having overlapping defects, cannot compensate each other in this way, so that stable hybrid molecules are not formed.

E. Recombination tests.

Once again the blue print for recombination tests was supplied by the phage geneticists and goes back to the year 1946. At a meeting at Cold Spring Harbour that year Hershey and Chase reported their first isolation of r and h phage mutants of the phage T2. At the same meeting Delbrück and Hershey presented another finding, which each had made independently, of the utmost importance to genetic research. Upon the infection of an E. coli with two or more phages that differed from each other in two genetic characters there issued from the infected cell some recombinant particles that had obtained one of these two characters from one parent and the other from the other parent. Thus Hershey carried out mixed infections with h and r mutants of T2 and found amongst the progeny some h+r+ wild type particles and some hr double mutant phages in addition to the two parental types. The h+r+ recombinant phage had derived its host

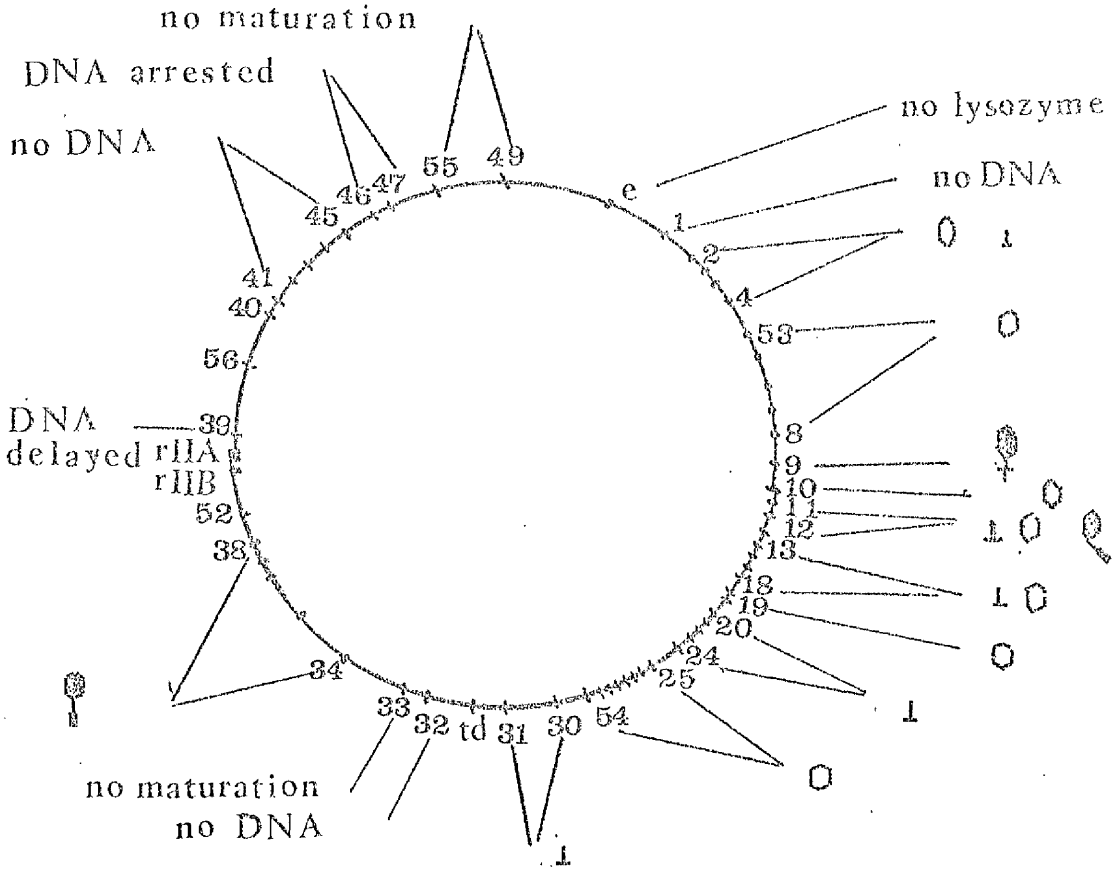
range character from the h+r parent and its rapid lysis character from the hr+ parent. The double mutant hr had derived its host range character from the hr+ parent and its rapid lysis character from the h+r parent. Thus it was discovered that phages could engage in genetic recombination, an activity once considered the prerogative of higher forms of life that had progressed from vegetative to sexual forms of reproduction, (Hershey, 1946; Hershey and Rotman, 1949). The essential feature of the recombination process is that the progeny, as well as containing the mutant parental virus also contains the wild type and double mutant recombinants in contrast to complementation where only parental types are produced.

While this work of mapping the h and r genes of T2 was going on Benzer decided to run one particular section of the map into the ground i. e. the r11 region. For this purpose Benzer crossed members of his r11 mutant collection 2 x 2 and selectively scored the frequency of r11+ wild type recombinants. In this way he hoped to detect very rare recombinant events between adjacent genetic sites since the limit of resolution of this method would allow him to find r11+ recombinants at an extremely low frequency. The frequencies of r+ recombinants produced in these very numerous crosses allowed Benzer to arrange all his mutants in a linear order and thus construct a very detailed map. Without going into detail Benzer's work showed that recombination was a process which could separate genetic sites represented by virtually contiguous nucleotides on the

phage DNA molecule.

Following from this, all the ts and amber mutants of T4 were arranged and ordered on a linkage map from recombination frequencies observed in a large number of genetic crosses. Crosses involving conditional lethal mutants were carried out under conditions permissive for the growth of each mutant. By plating under permissive conditions,the total progeny was measured and by plating under restrictive conditions the proportion of wild type recombinants was measured. From a large number of two and three factor crosses the map of T4 was constructed. It turned out that the genes which governed related phage functions appeared to lie in the same general area of the map. Furthermore the disposition of the genes appeared to be correlated with the time of intracellular phage growth at which the gene product makes its appearance or starts to function. The genes represented by conditional lethal mutants were distributed all over the genetic map. By 1966 a total of 66 genes concerned with conditional lethal mutants had been recognised and mapped. The following is the genetic map of T4.

Genetic Map of T4 (Edgar & Epstein 1965)



⊥ components present in defective lysates

Initially, using two-factor crosses, the most widely spaced loci appeared to show no linkage i. e. the chromosome could be best represented as a linear structure with two extremities. However a more sensitive test of linkage was provided by using three-factor crosses. By applying this test to the loci situated at the extremities of the T4 map Streisinger et al., (1964) detected unambiguous linkage between them and established that the sequence of the loci was that to be expected if the chromosome was really a continuous, or circular structure. The circularity was confirmed by using a very large number of ts and amber mutants in two-factor crosses.

One anomaly which arose in the mapping of T4, was the fact that the frequency of recombination in adjacent intervals was much greater than that expected from a random distribution i. e. the observed/expected number of double recombinants was greater than 1. It appeared therefore that a crossover between markers increased the chance of crossovers between closely adjacent markers. In other words negative interference was occurring. One explanation of negative interference is that effective pairing i. e. the kind of pairing which actually mediates recombination, is normally restricted to very small regions within which the probability of recombination is very high (Pritchard, 1955). On this theory, the occurrence of simultaneous recombination in two intervals requires an effectively paired region in both of them. If the genes are far apart these paired regions will arise randomly. On the other hand, if the genes are very

close together, then the two recombination events can arise within a single paired region, so that the probability of their simultaneous occurrence is no longer random, it is more frequent.

F. Two and three factor crosses.

The arrangement of mutations on a chromosome can be determined by means of crosses in which two mutations are followed - two-factor crosses. Consider the question of the arrangement of three mutant sites. The ordering of the mutations can be determined by three crosses in each of which two mutants are involved. A cross X between a+b and ab+ yields four progeny types: the two parental genotypes (a+b and ab+) and the two recombinant genotypes (a+b+ and ab). The cross Y between a+c and ac+ similarly gives the two parental types as well as the a+c+ and ac recombinants whereas the cross Z between b+c and bc+ produces the parental types and the recombinants b+c+ and bc. Each cross will produce a specific ratio of parental to recombinant progeny. Consider e. g. that cross X gives 10% recombinants, cross Y 3% and cross Z 8%. This suggests that markers a and c are most linked and that a and b are least linked. Therefore the genetic arrangement which best fits this data is

a c b

Although mapping by means of comparative recombination frequencies has been extensively used in the analysis of genetic fine structure and may be the only practicable method, its sensitivity to extraneous

sources of error as well as its inability to compensate for intrinsic variability in the recombination mechanism itself, such as that introduced by localised negative interference, renders it suspect when the distances between mutational sites become extremely small.

A standard method for determining the order of sites is by means of reciprocal three-factor crosses. When the three genes used in the above example are followed in the cross a+b+c+ x abc six different recombinant genotypes are found. These fall into three groups of reciprocal pairs. The rarest of these arises from the double crossover. By looking for the least frequent class it is possible to confirm or deny an arrangement postulated by two-factor cross data. If results of the cross a+b+c+ x abc were:-

a+ b+ c+ / a b c	a+ b+ c+ / a b c	15%
	a+ b c / a b+ c+	2%
	a+ b c+ / a b+ c	6%
	a b c+ / a+ b+ c	0.6%

Only if the order is a c b, does the fact that the rare recombinants are a+b+c and abc+ make sense. In other words, the three-factor cross data has confirmed the ordering suggested by the two-factor cross results.

G. Mechanisms of recombination.

For the mechanisms involved in recombination we must return again to the phage geneticists and to the work of Hershey on the r and h mutants of T2. From his initial recombination experiments Hershey concluded that the genetic material of phages consisted of linear arrays of genes, each gene carrying the hereditary information for some character of the virus, analagous to the genes in the chromosomes of higher organisms. He envisaged the process of recombination as occurring from crossing over events which lead to the reassortment of the genetic material. The chance of a crossover was a function of the distance separating two markers and thus the frequency of recombinants among the progeny gave a measure of the relative distances between markers; markers close together giving lower recombination frequencies than markers far apart.

However further study of genetic recombination in phage revealed that a phage cross does not represent merely the exchange of hereditary factors between two parents but that instead recombination involves the repeated interactions among the intracellular population of phage genomes descended from the infecting parents. Taking this fact into consideration Visconti and Delbrück (1953), formulated a theory of genetic recombination in phages to account for the recombinant frequencies obtained under various experimental conditions. This theory envisages that the vegetative phage genomes exist in an intracellular mating pool in which they undergo pairwise matings,

each mating leading to an exchange of genetic material by one or more crossovers between the two partners. Therefore the fraction of phages recombinant for two loci introduced into a cross depends not only on the linkage of the loci but also on the number of mating events that have occurred in the mating pool by the time that lysis of the infected bacterium has brought to term the intracellular growth process. Since linkage cannot therefore be simply equated to recombinant frequencies the true linkage of two genetic sites is defined as the average number of crossovers that take place at points between these sites in each mating of two vegetative phages in the mating pool: the greater the distance that separates the sites on the phage genome, the greater the average number of such crossovers per mating. But an observable recombination of genetic characters will result if, in any one mating an odd number of crossovers occurs at points between the genes determining these characters, for even numbers of crossovers no recombination would be manifested since the original configuration of the two characters is preserved. For very distantly linked genes the recombination frequency approaches the limit 0. 5. In other words if there occur a large number of crossovers per mating between two genes, half of the matings will end in an odd number and half in an even number of crossovers. For very closely linked loci the average number of crossovers per mating between the two loci is very much less than 1, and most crossovers will be single exchanges. Finally if the two markers are

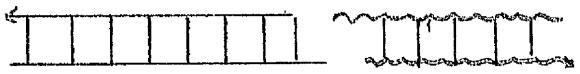
identical alleles there is no chance whatsoever that a recombinant is produced for two genetic characters that occupy exactly the same site of the phage genome.

These then are the overall rules governing recombination in phages, but what of events at the molecular level?

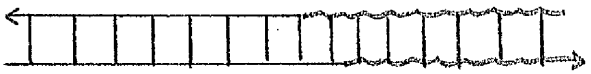
Hershey in 1948 discovered that contrary to the anticipations of exchange by crossover, (by analogy to the then known situation in higher organisms) the elementary recombination event generates only one but not both of the two complementary recombinants. It appeared that any given mating event produces either an hr or an h+r+ recombinant but not both types as would be expected from a reciprocal event. Hershey proposed that recombination may occur as an event incidental to the replication of the phage genome, a hypothetical mechanism that later came to be called copy choice. Under the copy choice doctrine a mating represents the coming together of two parental genomes at the moment when one of them happens to be serving as a template for the synthesis of a replicon. After part of the genetic information of the first parental genome has already been copied into the replica structure, the copy process suddenly switches to the second parental genome and begins to incorporate the genetic information of the second parent on to the growing daughter genome. A single recombinant phage thus arises which carries some of the genetic factors of one and some of the genetic factors of the other of the two mated parents. A further and entirely independent recombination act is required to produce the complementary type, which

would in no way be correlated with the formation of its complement, though it would occur with equal frequency. If there is a fixed probability per length of phage DNA that a copy choice switch will occur during a mating, then the chance that a replica genome recombinant for two genes would be produced still depends on the distance between the two loci. This then was the theory of the mechanism of recombination favoured up until and during the 1950's. The theory is however no longer held to be true. Besides accounting for the formation of only one of two complementary recombinants in a single act of genetic exchange, the copy choice mechanism entailed another more basic feature that distinguished it from an alternative theory of recombination (breakage and reunion) - recombinants are composed of newly synthesised DNA and should not contain any parental DNA. Meselson and Weigle (1961), using phage lambda were the first to show that phage recombinants do contain part of the DNA of the parental genomes that entered a cross and hence that genetic exchange involves breakage and reunion rather than as a result of copy choice. Their results from experiments using density labelled phages demonstrated unequivocally that discrete amounts of original parental DNA appear in recombinant phages, suggesting that recombination occurs by reassortment of the genomes of the partners in the mating event. Confirmation of this finding came from work by Tomizawa and Anraku (1964; 1965) on T4. E. coli were infected simultaneously with light ^{32}P-labelled and

heavy bromouracil-labelled T4. Cell metabolism was inhibited by the addition of potassium cyanide. Density gradient fractionation of extracted intracellular DNA revealed the presence of some ^{32}P-labelled DNA molecules of density intermediate between that of light and heavy (BU-labelled) DNA i. e. molecules containing material derived from both heavy and light parental genomes. Upon denaturation, the heavy and light components of the molecules separated and the P^{32} label assumed the density of light DNA. These molecules of intermediate density thus appeared to be composed of one P^{32} labelled light DNA segment and BU labelled heavy segment joined by hydrogen bonds, but with gaps.



Joint molecule



Recombinant molecule

Thus it appeared that a "joint" DNA molecule that is likely to be an intermediate in the genesis of genetic recombinants can be formed while protein and nucleic acid synthesis are severely reduced. The experiment was repeated using in the place of cyanide, FUdR. This causes a great reduction in the rate of DNA synthesis although

protein synthesis proceeds at nearly the same rate. Formation of "joint" ^{32}P-BU labelled phage DNA was observed also under these conditions. However whereas almost all of the joint molecules detected after incubation of the infected cells in BUdR for twenty minutes or less were of the same hydrogen bonded type as those formed in the presence of cyanide, many molecules present after incubation for 45 minutes in FUdR appeared to be made up of segments of ^{32}P labelled light and BU labelled heavy parental DNA bound in covalent linkage, since the heavy and light components could no longer be separated by melting the double helix. It was concluded that the slow progress of DNA synthesis in the FUdR treated phage infected cells seemed to suffice to convert the hydrogen bonded "joint" molecules representing nascent recombinants into the covalently linked DNA continuum in the form of which the phage genome matures in the infective progeny particle. Further experiments with parental phages carrying various amber mutations which fail to synthesise phage DNA in non-permissive host bacteria showed the appearance only of "joint" molecules. This gave further support to the theory that phage DNA synthesis is required to establish covalent linkage between polynucleotide chains of mixed parental origin.

H. Heterozygotes.

Hershey and Chase (1951) observed that the progeny from T2r x T2r+ crosses produced plaques not only of the parental type

i.e. small turbid plaques and larger clear plaques but also of a mixed morphology. These plaques had a mottled appearance being composed of zones of lysis inhibition interspersed with zones of clearing. About 2% of the progeny plaques from r x r+ crosses had this mottled appearance. When the particles contained in these mottled plaques were replated in turn they gave rise to about equal numbers of r and r+ plaques and again about 2% of mottled plaques. Since it was shown that the mottled plaques were initiated by infection with single phage particles, it was concluded that these particles must carry both the r and r+ alleles and were therefore heterozygous for the r region. The essential features of heterozygotes are as follows:- (1) They are not restricted to any pair of alleles but are found to arise with the same frequency among the progeny of a cross with respect to every locus examined. (2) For crosses between phages differing by two loci, some heterozygotes are heterozygous for both loci and some are homozygous for one locus and heterozygous for the other. (3) The heterozygous region is small being about 10 map units long or about 1% of the length of the chromosome. (4) For crosses involving phages which differ by three loosely linked loci, particles selected as being heterozygous for the middle marker are frequently recombinant for the outside markers. (5) The frequency with which heterozygotes are made remains constant under different conditions, i.e. heterozygotes are being continually formed in the mating pool but are lost at the same

rate as a result of segregation. In other words heterozygotes may represent a normal stage in the production of recombinants and those which are observed among the progeny particles are a proportion which are trapped by maturation before they can segregate. The models proposed to explain the formation of heterozygotes are examined in the Discussion Section.

III. Animal Virus Genetics.

In the absence of evidence to the contrary, it must be supposed that the genetic mechanisms of animal viruses are governed by the same general rules which apply to bacteriophage and so it would appear that the phage geneticists have laid down the master plan for workers embarking on the field of animal virus genetics. Despite this background wealth of knowledge pioneered by the phage geneticists, animal virus genetics is still in its infancy. Although temperature-sensitive mutants of many RNA and DNA animal viruses have been isolated, relatively little work has been done to study genetic recombination and to construct linkage maps. The first successful search for conditional lethal mutants of an animal virus was initiated by McClain (1965) when it was discovered that a proportion of the u mutants of rabbitpox virus (Gemmell and Fenner, 1960) were host dependent, in that they failed to replicate in PK cells, a continuous line of cells derived from pig kidney. By 1963/64 when Edgar's work became widely known, the idea of using conditional lethal mutants for the analysis of the genetics and physiological behaviour of animal viruses was taken up by several workers. Cooper (1964) isolated ts mutants from Polio virus Type 1: Roizman and Aurelian (1964) studied the behaviour of HSV in a restrictive host cell (dog kidney) and a mutant of it able to grow in these cells, and Burge and Pfefferkorn (1965) began their studies of ts mutants of Sindbis virus. Following these initial isolations, investigations of

ts mutants of Semliki Forest virus were initiated and ts mutants of Influenza virus were isolated and used for genetic studies (Mackenzie, 1968). This then was the beginning of research in animal virus genetics. However the full potential of conditional lethal mutants of animal viruses has not yet been realised. Almost all investigations have been concerned with ts mutants, since no animal cell has yet been recognised as containing a nonsense suppressor. Therefore the most useful class of conditional lethal i. e. amber mutants cannot be used.

The isolation of temperature sensitive mutants has been reported for a large number of both RNA and DNA viruses. It is beyond the scope of this introduction to go into detail of all the work which has been reported. It has been decided therefore to summarise the experiments undertaken with certain viruses in different groups and to go into detail only where it is thought necessary.

A. RNA Viruses.

(1) Encephalovirus group.

Temperature sensitive mutants have been isolated from Sindbis virus and Semliki Forest virus and the work carried out with these viruses has been of a similar nature. Burge and Pfefferkorn (1966) isolated 23 ts mutants of Sindbis virus and they were assigned as RNA +ve or RNA -ve. The RNA +ve mutants were found to be more heat labile than the RNA

-ve mutants or the parent virus at 60°C. Recombination could not be demonstrated but complementation was shown to occur and was studied in some detail (Burge and Pfefferkorn, 1966; Pfefferkorn and Burge, 1967). The complementation yields exceeded the single parent infections by factors of 3-300, though the efficiency relative to wild type infection, was low. Complementation efficiency was found to be unaffected by the m. o. i. and the time course of production of virus from complementation tests followed that of the growth of wild type virus. The RNA +ve mutants were assigned to three complementation groups corresponding to alterations in their physiological response. At the restrictive temperature one group failed to form nucleocapsids, but did form viral envelope proteins. The second group exhibited the reverse effect and those of the third group were defective in a protein necessary for virus maturation. (All the RNA -ve mutants were found to be defective in the synthesis of polymerase) (Burge and Pfefferkorn, 1968; Strauss et al., 1968; Scheele and Pfefferkorn, 1970).

With Semliki Forest virus, 38 ts mutants were obtained following mutagenesis and although the mutants were shown to complement, it was not as apparent as with Sindbis virus and has not proved useful for grouping mutants. Recombination has been demonstrated between the mutants (Sambrook, 1965). Mutants were found which were defective in structural components,

maturation factors and polymerase activity (Tan et al., 1969).

(2) Paramyxo group.

There have been reports of the isolation of ts mutants from Respiratory syncytial virus (RSV) and Newcastle Disease virus (NDV). Ts mutants of RSV were isolated following mutagenesis with 5-fluorouracil. Suppression of growth from 16-fold to complete suppression was found at the restrictive temperature and three of the mutants were found to affect late functions. The ts mutants of RSV were isolated following the discovery that ts mutants of Semliki Forest virus and Reo virus were less virulent in experimental laboratory hosts. It was thought that if the ts mutants of RSV could be isolated which would grow well at the temperature of the nasopharynx (31/32°C), but not at the temperature of the lower respiratory tract (37°C), then they would be potentially useful in immunization,(Gharpure et al.1969

With NDV Dahlberg and Simon (1968) obtained 48 nitrous acid induced ts mutants. So far complementation tests have revealed nine non-overlapping groups, of which one group contains 16 mutants. The recombination reported for NDV now seems to result from the production of heteroploid virus particles (Dahlberg and Simon, 1969).

(3) Myxo virus group.

Although a great deal of the early work in animal virus genetics was carried out with Influenza virus, it is only

comparatively recently that two groups of workers have begun systematic studies with conditional lethal mutants of this virus. Simpson and Hirst (1968) and Mackenzie (1968) working independently have isolated a large number of ts mutants of Influenza A virus. Both complementation and recombination have been demonstrated by Simpson and Hirst. On the basis of high recombination frequencies and the isolation of several pieces of viral RNA from the virion, they postulated that the genome may consist of 5 pieces of RNA which replicate separately and undergo assortment as separate segments of RNA. This is in direct contrast to the work of Mackenzie, who found that the recombination frequencies obtained with 16 ts mutants were additive, though high and on the basis of this he has postulated a single linear genome.

At the physiological level, certain of the mutants isolated by Mackenzie were found to be defective in haemagglutinin, others in neuraminidase and two were found to be defective in structural proteins. All the mutants were found to be less virulent than the parent virus. Most were found to be completely non-lethal, although all of them immunised infected mice against a challenge dose of virulent wild type virus (Mackenzie and Dimmock, 1972).

Recent experiments which would tend to support the theory of Simpson and Hirst, have been reported by Tobita (1971).

Using two strains of Influenza A virus, it was found in two-factor crosses testing for the serotypes of haemagglutinin and neuraminidase, plaque size, plaque forming ability in FL cells and the requirement for DEAE dextran for plaque formation, that the recombination frequencies ranged from 18% to 43%. There was no evidence of linkage between the markers and from the absence of recombination within the serotype of the haemagglutinin or that of the neuraminidase, it was suggested that recombination occurs mainly by the exchange of genome pieces. Taken together with the broad genetic compatability which was found as well as high recombination frequencies, it was suggested that these findings supported the proposal for physical discreteness of functional units and independent assortment of these units during the process of maturation.

(4) Rhabdo virus group.

The isolation of ts mutants of Vesicular stomatitis virus has been reported from a number of sources (Flamand, 1969; Lafay, 1969; Lafay and Berkaloff, 1969; Holloway et al., 1970; Pringle, 1970). Pringle initially isolated 75 ts mutants of the Indiana strain of VSV and these were assigned to four complementation groups on the basis of qualitative tests. Some of the mutants were used to obtain quantitative complementation data. No recombination was observed within Groups I and Iv, whereas 0.31-3.4% recombinants were observed between the groups.

The lack of evidence of linkage was expected due to the small size of the genome and it was suggested that the cistrons may be physically discrete and reassortment of sub-units was suggested rather than true genetic recombination.

Flamand (1969) identified 5 complementation groups using spontaneously arising ts mutants and reciprocal complementation studies revealed four groups in common with the Glasgow mutants (Flamand and Pringle, 1971). At the restrictive temperature mutants of Groups 1 and 1v did not induce synthesis of viral RNA, those of Group 3 did. The functions associated with the mutants of Groups 1, 11 and 1v were shown to be required early, those of group 111 were not required until a later stage of the viral cycle (Pringle and Duncan, 1971).

A further 48 ts mutants of the New Jersey strain of VSV have been isolated and classified into 6 complementation groups. No complementation has been observed between these and the mutants of the Indiana strain (Pringle et al., 1971).

(5) Picorna virus group.

(a) Foot and Mouth Disease virus (FMDV).

It had been shown in 1965 that recombination could be obtained following mixed infection of cells with immunologically distinct strains of FMDV (Pringle, 1965). Following this ts mutants were isolated which were shown to complement and recombine (Pringle, 1968). Further

work examined the physiological defects of 17 ts mutants in terms of their ability to produce infective RNA and complement fixing antigen under restrictive conditions. Genetic recombination was observed in crosses of mutants with different physiological effects, such that the mutants were arranged in five groups, but the problems involved in interpretation of two factor cross results were stressed (Pringle et al., 1970).

(b) Polio virus.

Studies with conditional lethal mutants of riboviruses have progressed further with Polio virus than with any other virus. This followed the isolation in 1963 of a large number of ts mutants following mutagenesis with 5' fluorouracil (Cooper, 1964). Following the approach with T4, it was shown that when cells were mixedly infected with two of the mutants at the non-permissive temperature, the yields were 4-14 times the sum of the yields of each mutant when grown separately. The yields were found to be 90-99% mutant but only one of the mutants was detected in the yields (Cooper, 1965). This was followed in 1966 by an attempt to determine the number and function of the defective viral genes in terms of (1) The production of Polio virus antigen: a+, (2) The prevention of cellular incorporation of thymidine: pti+, (3) The prevention of P^{32} incorporation

into respectively 28s, 16s or 4-10s cellular RNA: ppi_1^+, ppi_2^+, ppi_3^+. The wild type virus displayed all four characters at the non-permissive temperature but most of the 29 ts mutants tested did not. Four groups were defined with the following combination of characters - Group A: a, ppi_3, pti; Group B: a, ppi_3; Group C: a; Group D: all characters wild type. Other combinations were not found and so it was concluded that these characters covaried in an asymmetrical or polar fashion (Cooper et al., 1966).

In recombination studies, as there was no published precedent for a qualitative map obtained by recombination analysis of a virus growing in animal cells or of an RNA virus growing in any cell, particular attention was paid to possible causes of variability in recombination frequencies:- (1) Care was taken to identify double mutants since multiple mutants are common with 5 FU mutagenesis. (2) Single step mutants were selected which were relatively non leaky. (3) Disaggregation of virus stocks was achieved by using glycine buffer and (4) Assay procedures were standardised. The mean recombinant frequencies obtained were then reproducible and characteristic of each pair of mutants, being between 5.2 and 31.4 times the total background reversion rates and ranging from 0.02% to 0.85%. Certain

genetic sequences were proved by three factor crosses involving a mutant adapted to guanidine resistance, and these showed that recombination frequencies were additive. Recombination was shown to be non-random with time, most of the mating events occurring early in the growth cycle (Cooper, 1967; 1968).

The work then progressed to a physiological level with tests for the production of infectious RNA and serum blocking antigen (Wentworth et al., 1968). No mutant produced fully wild type yields of either RNA or antigen. On the basis of the previous classification into groups, it was shown that Group A produced the least RNA and antigen while Group D produced the most. Mutants of Group B and Group C produced intermediate yields. Work of structural proteins showed that 29 ts mutants lost infectivity at 45° more rapidly than ts+ and were concluded as having defects in virus structural proteins. A division into structural and non-structural proteins accorded very closely with their position on the genetic map, enabling the region of the map specifying structural proteins to be defined McCahon and Cooper, 1970). Seven different structural proteins of Polio virus have been distinguished so far. As these exceed the probable translational capacity of the virus genome for such proteins, it is proposed that there

are multiple cleavage sites in the precursor protein(s) and that these are used in varying combinations in different viral strains (Cooper et al., 1970).

(6) RNA tumour viruses.

The isolation of two ts mutants of avian sarcoma virus has been reported by Vogt et al., (1970). The mutants were found to be ts with respect to virus-induced cellular transformation as well as virus production. The ts function of one mutant was found to occur early in the infectious cycle and the other mutant was shown to have a late function. Coinfection of cells with one ts mutant and a wild type virus led to complementation. The wild type could be distinguished from the mutant by a number of markers. It was necessary to carry out the complementation experiments in this way because the two ts mutants both belonged to the same avian tumour virus subgroup and therefore interfered with each other.

Temperature sensitive mutants of the Schmidt-Ruppin strain of Rous Sarcoma virus isolated by Martin (1970) have been shown to transform at the permissive temperature but not at the non-permissive temperature although the virus grew normally at both temperatures. When shifted from the permissive to the non-permissive temperature, the infected cells lost the properties of transformed cells, indicating that the mutant function was required for the maintenance of the transformed state. The

mutants were also found to produce fusiform cells instead of the round cells resulting from infection by the wild type virus.

The observation that the viral genome was required for the initiation and maintenance of neoplastic cellular properties would suggest that further work on ts mutants of RNA tumour viruses may lead to an identification and characterisation of viral functions responsible for neoplastic transformation.

B. DNA Viruses.

(1) Pox virus group.

Two members of this group, rabbitpox and vaccinia, have been studied. Rabbitpox, with a DNA molecule of 160×10^6 daltons, could code for four hundred average sized proteins (about 80,000 amino acids) so that the complete genetic and functional analysis rendered theoretically possible by the use of ts mutants of this virus would be a major undertaking. The first ts mutants were isolated in 1966 (Sambrook et al., 1966). This was followed by the examination of 18 ts mutants in complementation and recombination experiments and for a few physiological characters. All the mutants except one produced u+ pocks on the chorioallantoic membrane. All the mutants displayed the same inactivation kinetics as the wild type and all synthesised DNA at the restrictive temperature. All except three produced the same range of soluble antigen at the restrictive

and permissive temperatures. Recombination tests showed that all the mutants were different from each other, and the complementation tests suggested that the ts defects in most of the mutants were probably in different cistrons. An ordered arrangement of the ts mutants was unable to be made due to the lack of another marker for three factor crosses (Padgett and Tomkins, 1968). However previous experiments with u mutants (white pocks on chorio allantoic membrane) of the virus showed that 18 mutants could be arranged in a linear order according to the presence or absence of u+ pocks (ulcerated on C. A. M.) in a series of pairwise crosses. Five of the mutants were assigned to one linkage group, eleven to another and two of the mutants could not be assigned to either group. Recombination frequencies were not calculated due to certain difficulties and technical problems which were encountered, (Gemmel and Fenner, 1960). Further experiments with p mutants (failure to multiply on PK-2a cells) assigned 34 p mutants to one linkage group of the recombination matrix of u mutants and by 1966, the linkage map of the u mutants had been extended to include 51 mutants.

A number of ts mutants of Vaccinia virus have been isolated. Some have been shown to be heat labile and one mutant has been studied in detail. This mutant was shown to have a delayed and decreased rate of DNA replication as well as a diminished rate of messenger RNA transcription at both the permissive and

restrictive temperatures. Temperature shift experiments confirmed that it was an early function mutant. It was shown to have only half the RNA polymerase activity associated with the wild type virus and the DNA polymerase was twice as heat labile as the wild type. It was found to complement weakly but to recombine efficiently with two other ts mutants (Basilico and Joklik, 1968).

(2) Papovavirus group.

With viruses with genomes as small as those of Polyoma and SV40 conditional lethal mutants, obviously afford an opportunity for totally mapping the genome and elucidating the functions of all the genes of these viruses, a prospect rendered particularly attractive by the oncogenic potential of these viruses. The difficulty of such investigations lies in the prolonged incubation needed to produce plaques, or transformation at the low temperature if ts mutants are sought.

Temperature shift experiments with one ts mutant of Polyoma virus showed that it was ts in transforming ability as well as plaque formation. However once a cell was transformed by this mutant at the low temperature it retained its transformed character when cultivated at an elevated temperature. The explanation was that the ts mutant was sensitive to high temperature when replicating autonomously but insensitive when integrated into

the host genome (Fried, 1965a; b). Since that time Eckart (1969) and DiMayorca (1969) have isolated a large number of ts mutants of Polyoma virus. About half of the mutants were shown to complement in mixed infections. Two complementation groups were defined, one of which was defective in a structural protein and the other in a function required for DNA synthesis. Most of the DNA -ve mutants were shown to fail to cause transformation at the restrictive temperature. All the mutants were found to produce T antigen at the restrictive temperature. Recombination has been demonstrated between two ts mutants of Polyoma virus (Ishikawa and DiMayorca, 1970). The assay was shown to be complicated by complementation and leakiness but when the necessary controls were introduced, the recombination frequency was found to be at least 0.24% between the pair.

As well as the work on Polyoma virus there have been reports on the isolation of ts mutants of SV40 virus. One ts mutant of SV40 was studied in terms of the synthesis of viral DNA and viral induced proteins at the restrictive temperature (Takemoto and Martin, 1970). A second report by Tegtmeyer and Ozer (1971), on the characterisation of 10 ts mutants, showed that the mutants could be divided into three functional and two complementation groups. Seven mutants produced T antigen, infectious DNA and structural antigen but predominately the empty shell type of virus particle. Two mutants produced T antigen and infectious DNA

but no V antigen or virus particles were found. A single mutant was defective in the synthesis of viral DNA, viral structural antigens and viral particles. This mutant stimulated cell DNA synthesis at the restrictive temperature and complemented the other two functional groups of mutants.

(3) Adeno virus group.

The first experiments on conditional lethal mutants of Adeno virus were on Adeno virus 12, where it was discovered that mutants of the virus, which produced clear plaques (cyt) could be recovered from wild type virus (Takemori et al., 1968). The cyt mutants were found to be less oncogenic (except one) than cyt+, and it was discovered that a proportion of the mutants replicated very poorly in a particular line of KB cells (Takemori and Aldrich, 1968). Although it has been found that different cyt kb mutants fail to complement in the restrictive line of KB cells, the system does provide a host cell which is restrictive for certain mutants, which can therefore be regarded as host dependent conditional lethal mutants.

A number of mutants of Adeno virus 31 have also been isolated and one of these mutants has been studied in some detail. It was shown that at the non-permissive temperature, T antigen, DNA polymerase and thymidine kinase were induced but viral DNA and capsid proteins were not synthesised (Suzuki and Shimoto, 1971). The isolation of ts mutants of avian Adeno virus

has also been reported (Ishibashi, 1970).

The most comprehensive analysis of Adeno virus genetics is that of Adeno virus 5. Temperature sensitive mutants were isolated using nitrous acid, hydroxylamine and 5-bromodeoxyuridine (Williams et al., 1971). Complementation analysis has assigned ten of the mutants to nine groups and the complementation was shown in most cases to be extremely efficient. Preliminary recombination experiments gave recombination frequencies ranging from 0.5 to 7.6% and it was shown that true ts+ recombinants were being produced, (Williams and Ustacelebi, 1971). Two of the ts mutants transform rat embryo cells, one with the same frequency as the wild type and the other with a considerably lower frequency (Williams and Ustacelebi, 1971). Two of the mutants which complement each other in their ts function, do not induce interferon at the non-permissive temperature but induce it at the permissive temperature (Ustacelebi and Williams, 1972).

(4) Herpes virus group.

For HSV recombination with markers affecting pock character and virulence were reported by Wildy (1955), but until recently little further progress has been reported. The isolation of ts mutants of HSV was first reported by Subak-Sharpe (1969) and since that time the isolation of ts mutants of a different strain of HSV Type 1 has been reported by Schaeffer et al., (1971)

and the isolation of ts mutants of HSV Type 2 has been reported by Timbury (1971). The isolation of ts mutants of one other member of the Herpes group - Pseudorabies virus has been reported by Huy et al., (1971) and Pringle (1972).

It can be seen from the foregoing account that the number of investigations using conditional lethal mutants to study animal viruses is substantial. However, with animal viruses the study of genetic recombination is in its infancy and the establishment of linkage maps has hardly begun. In general the technical problems peculiar to different virus systems makes quantitation for assigning the order of mutants difficult. Genetic maps have not been forthcoming and at present the genetic map of Polio virus is the only reasonably complete map.

Since the time of the first isolation of ts mutants of Glasgow strain 17 we have made a genetic study with nine ts mutants and the results obtained are reported in this thesis.

MATERIALS AND METHODS.

1. Virus.

Herpes simplex virus Type 1 - Glasgow strain 17 was used throughout this study. This strain was derived by plaque purification from an isolate made from a patient by Dr. Connie Ross at Ruchill Hospital, Glasgow. The virus grows well at temperatures in the range 31° to 38° and produces plaques with a non-syncytial morphology. From this wild type virus a mutant with a syncytial plaque morphology (designated syn) was isolated, purified by 3 successive single plaque purifications and made into 17 syn stock. This 17 syn stock was the parent from which nine independently induced temperature sensitive (ts) mutants were derived following mutagenesis with 5-bromodeoxyuridine. Each ts mutant was given a letter symbol, eg. tsA. The syn and ts syn mutants were isolated by Professor J. H. Subak-Sharpe.

2. Tissue culture cells.

The cells used throughout this study were the continuous line BHK21/C13 of hamster kidney fibroblasts isolated by Macpherson and Stoker (1962).

3. Media.

(a) Growth media.

The basic growth medium was a modified Eagle's medium

(Busby, House and Macdonald, 1964) containing 100 units/ml of Penicillin, 100 μg/ml of Streptomycin, 0.002% phenol red and 0.2 μg/ml of the antimycotic agent n-butyl, p-hydroxy benzoate and supplemented with Difco tryptose phosphate broth (stock solution of 2.95% in distilled water) and serum in the following combinations by volume:

E.T.C. 80% Eagle's medium, 10% tryptose phosphate broth, 10% calf serum.

E.T.C. 5%. 85% Eagle's medium, 10% tryptose phosphate broth, 5% calf serum.

E.T.Hu. 80% Eagle's medium, 10% tryptose phosphate broth, 10% pooled human serum.

(b) Phosphate buffered saline A (P.B.S.A.).

A solution of 0.17 M-NaCl, 3.4 mM-KCl, 10 mM-Na_2HPO_4 and 2 mM-KH_2PO_4, pH 7.4 (Dulbecco and Vogt, 1954).

(c) P.B.S.A.C.

P.B.S.A. supplemented with 10% calf serum.

(d) Trypsin-versene.

One volume of 0.25% (w/v) Difco trypsin (dissolved in tris-saline) and four volumes of 0.6 mM versene.

(e) Versene.

Ethylene diaminetetraacetic acid (0.6 mM) dissolved in P.B.S.A. and containing 0.002% (v/v) phenol red.

(f) Formal saline.

A solution of 4% (v/v) formaldehyde in 85 mM-NaCl, 0.1 M-Na_2SO_4.

(g) Giemsa stain.

A 1.5% (v/v) suspension of Giemsa in glycerol, heated at 56° for 90-120 minutes and diluted with an equal volume of methanol, (Dacie, 1956).

4. Cell culture system.

Low passage BHK21/C13 cells were seeded in 200 ml of E. T. C. medium in rotating 80 oz. Winchester bottles (10^7 cells/bottle) and incubated in an atmosphere of 5% CO_2 at 37° on a roller bottle culture rack of the type described by House and Wildy (1965). Two to three days later when a confluent monolayer had formed, ($3-4 \times 10^8$ cells/bottle) the cells were removed from the walls of the bottle by washing twice with Trypsin-versene, suspended in E. T. C. and dispensed into roller bottles at approximately 2×10^7 cells/bottle. The cell line was subsequently maintained by similar serial dilution. Roller bottles containing confluent cell monolayers produced by this method were used for all virus growth experiments.

5. Production of virus stocks.

The growth medium was removed from roller bottle cultures of newly confluent BHK21/C13 cells and replaced by 20 ml E. T. C. 5%

containing virus at a multiplicity of 0.003 plaque forming units (p.f.u.)/cell. The virus inoculum was allowed to adsorb to the cells for one hour at 37° as the bottle rotated. After adsorption 50 ml E.T.C. 5% medium was added and the bottle returned to the roller rack to be incubated at 31° for 66 hours or until confluent cytopathic effect was visible. After this time, the cells were harvested by shaking the cell sheet into the medium, the excess medium being removed by centrifugation at 900 x g for 10 minutes at 4°C. The virus containing cell pellet was resuspended in a small volume of supernatant and the cells disrupted by sonication for approximately 5 minutes at 60 m amperes in a Dawe Soniclean Generator. After centrifugation at 900 x g the supernatant which contained the virus particles was removed to 4°C while the debris was resuspended in a small volume of the supernatant and the extraction repeated. The supernatant fractions were pooled and subsequently stored at -70° for use as viral inoculum. The supernatant obtained from the initial centrifugation was also used when the titre of this extracellular virus was high enough. This procedure was used for the growth of all virus stocks. The usual yield of virus was 100 p.f.u./cell and the E.M. visible particle:p.f.u. ratio was less than 10:1.

6. Plaque titration.

Dilutions of virus in E.T.C. or P.B.S.A.C. were added to suspensions of cells at 4 x 10^6 cells/ml. The infected cells were

maintained in suspension for 30 minutes at 37° to allow adsorption (and some penetration) to occur and then aliquots of 4 x 10^6 cells were dispersed in 4 ml of E. T. Hu. in 50 mm plastic plates. After incubation for two days at 36° or 38° or for three days at 31°, the medium was removed and the cultures fixed by exposure to formal saline for 10 minutes. After removal of the formal saline the cell sheet was stained with Giemsa for 10 minutes at room temperature. The plaques were counted using a dissecting microscope at a magnification x 50.

7. One-step growth curve.

BHK21/C13 cells suspended in E. T. C. at a concentration of 4 x 10^6 cells/ml were infected with virus at a multiplicity of 5 p.f.u./cell. After absorption in suspension at 37° for one hour or adsorption overnight at 4°, the cells were pelleted at 900 x g for 10 minutes, the supernatant removed and replaced by fresh warm E. T. C. The cells were dispensed into plastic plates (4 x 10^6 cells/plate) in 4 ml of E. T. C. and replicate plates incubated at 31°, 36° and 38°C. At subsequent intervals sets of plates incubated at all three temperatures were removed from the incubators and the cells were suspended in growth medium and sonicated. The liberated cellular virus was titrated for infectivity to determine the total infectious virus produced.

8. Single plaque purification of virus.

Purification of virus stocks was achieved by growing stocks

from single plaques. Cells were infected with virus at dilutions calculated to give very few plaques per plate. After incubation in E. T. C. at 31° for 3 days, the medium was removed and the monolayer washed with fresh E. T. C. to remove any extracellular virus. Using a dissecting microscope the virus from single non-overlapping plaques was taken up with finely drawn out pasteur pipettes and each transferred to 1 ml of E. T. C. in a bijoux bottle. Virus was released by sonication for approximately 1 minute. The 1 ml of virus was added to 4 ml of cells and after adsorption in suspension 1 ml aliquots were dispensed in 4 ml E. T. C. into 50 mm plastic plates. After incubation for 2/3 days the same procedure was repeated. Single plaque purification was successively carried out three times. From the final plating single plaques were again isolated into 1 ml medium. This was added to 1 ml of cells (4×10^6 cells/ml) in E. T. C. and after adsorption the cells were plated out and incubated. Confluent cytopathic effect appeared in about 24 hours when the cells were harvested and the virus released by sonication for approximately 5 minutes. This virus stock was adsorbed to 5 ml of cells (4×10^6 cells/ml) and the titre increased by growth for approximately 24 hours in a 20 oz flat bottle containing 20 ml E. T. C. The resulting virus was released by sonication and used as seed for further stocks using the roller bottle culture method.

When purification is being carried out to obtain stocks of pure plaque morphology, the plaque forming units in the lysate at each stage, are estimated by the standard assay to ascertain if the plaques

obtained are of a single type.

9. Complementation tests for HSV temperature sensitive mutants.

(a) Infectious centre test.

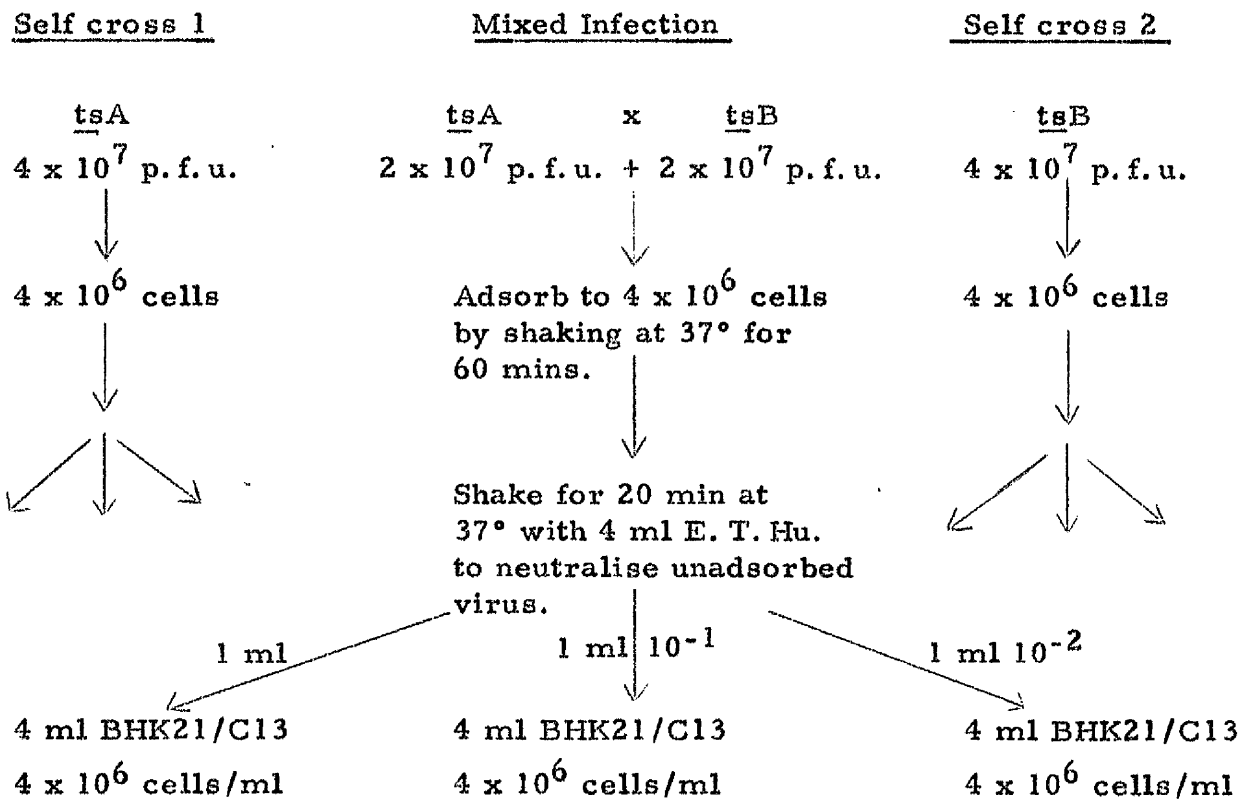
4×10^6 cells in 4 ml E. T. C. were infected at a multiplicity of exposure of 5 p.f.u. /cell with each of two ts mutants (total M. O. I. = 10). After adsorption in suspension for one hour at 37°, 4 ml of E. T. Hu. was added to each test to neutralise unadsorbed virus. This was done by further shaking at 37° for twenty minutes. Three dilutions of infected cells were then added to three sets of 4 ml of uninfected carrier cells at a concentration of 4×10^6 cells/ml, and the cells were then dispensed into 50 mm plastic petri dishes - 1 ml of cells plus 3 ml E. T. Hu. From each dilution of the initially doubly infected cells four plates were obtained - two plates were incubated at 38°, one at 36° and one at 31°C. After incubation for two days at 36° and 38° and three days at 31°, the cells were fixed and stained and the infectious centre plaques were counted. Self cross controls were carried out in the same manner with a total multiplicity of exposure of 10 p.f.u. /cell.

The scheme for the experiment is illustrated in Table 1.

The complementation index (C. I.) was calculated as -

$$C.I. = \frac{(A+B)npt/pt}{\frac{1}{2}(Anpt/pt + Bnpt/pt)}$$

Table 1. Complementation Test: Infectious Centre Assay



From each dilution 4 plates.

2 incubated at 38° } 2 days
1 incubated at 36° }
1 incubated at 31° - 3 days } - Fix and stain plates and count plaques

Where A and B represent any pair of ts mutants and npt/pt is the ratio of infectious centre titres at the non-permissive (36° or 38°) and permissive (31°) temperatures respectively. The denominator is halved because the control infected cells received twice the number of p. f. u. of a mutant compared to the mixed infection. Where no plaques at all were observed at the non-permissive temperature the highest dilution used was given the value 1.

(b) Yield experiment.

As in the infectious centre test 4×10^6 cells in 4 ml of E. T. C. were infected at a multiplicity of exposure of 5 p. f. u. / cell with each of two ts mutants. After adsorption in suspension for one hour at 37°, excess virus was neutralised by the addition of 5 ml E. T. Hu. and further shaking for twenty minutes at 37°C. The infected cells were then pelleted by centrifugation at 900 x g for ten minutes and the medium discarded. The cell pellet was resuspended in 4 ml E. T. C., plated and after incubation for twenty hours at 38° the cells and the medium were harvested. Virus was released from the cells by sonication for approximately one minute. The plaque forming units in the lysate were estimated by the standard plaque assay at 31° and 38°C. Self crosses were carried out in the same way with a total multiplicity of 10 p. f. u. /cell. The scheme for the experiment is illustrated in Table 2.

Table 2. Complementation Test: Progeny Yield Test

Self cross 1	Mixed Infection	Self cross 2
tsA	tsA x tsB	tsB
4×10^7 p.f.u.	2×10^7 p.f.u. + 2×10^7 p.f.u.	4×10^7 p.f.u.
↓	↓	↓
4×10^6 cells	Adsorb to 4×10^6 cells by shaking at 37° for 60 min.	4×10^6 cells
↓	↓	↓
	Shake for 20 min at 37° with 4 ml E. T. Hu. to neutralise unadsorbed virus.	
↓	↓	↓
	Pellet cells, discard medium and resuspend in 4 ml E. T. C. and plate.	
↓	↓	↓
	Incubate 20 hr at 38°	
↓	↓	↓
	Harvest cells + medium and sonicate.	
↓	↓	↓
	Titrate virus at 31° and 38°.	

The complementation index was calculated as -

$$C.I. = \frac{(A+B)^{31°} - (A+B)^{38°}}{\frac{1}{2}(A^{31°} + B^{31°})}$$

Where $(A+B)^{31}$ and $(A+B)^{38}$ represent the titre of progeny from the cross tsA x tsB, performed at 38°, when assayed at 31° and 38° respectively. The denominator represents the sum of the yields of the control infections and is halved for the reason described above (Methods Section 9a). The $(A+B)^{38}$ factor corrects for any ts+ recombinants that may have arisen. When self crosses exhibited no growth at the n.p.t., the value of 1 was assumed for the denominator.

10. Recombination tests.

(a) Two factor crosses.

4×10^6 cells in 4 ml of E. T. C. were infected at a multiplicity of exposure of 5 p. f. u. /cell of each of two ts mutants. After adsorption in suspension at 37° for one hour, the excess virus was neutralised by the addition of 4 ml E. T. Hu. and further shaking for twenty minutes at 37°C. The cells were pelleted by centrifugation at 900 x g for ten minutes, the medium discarded and the cells resuspended in 4 ml E. T. C. The infected cells were dispensed in 50 mm plastic dishes and incubated at 31° for twenty four hours. After incubation the cells and the medium were harvested and the virus released from the cells by sonication for 1 minute. The plaque forming units in the lysate were estimated by the standard plaque assay at 31° and 38°C. Self crosses were carried out in the same way with a total multiplicity of exposure of 10 p. f. u. /cell.

The scheme for the experiment is illustrated in Table 3.

Recombination frequency (R. F.) was calculated as -

$$\text{R. F. \%} = \frac{(A+B)^{38°}}{(A+B)^{31°}} \times 2 \times 100$$

Where $(A+B)^{38°}$ represents the titre of progeny from the cross tsA x tsB assayed at 38°. The factor of 2 takes into account the double mutant recombinant which is assumed to arise with the same frequency as the selected ts+ recombinant.

Table 3. Recombination Experiment

Self cross 1	Mixed Infection	Self cross 2.
tsA	tsA x tsB	tsB
4×10^7 p.f.u.	2×10^7 p.f.u. + 2×10^7 p.f.u.	4×10^7 p.f.u.
↓	↓	↓
4×10^6 cells	Adsorb to 4×10^6 cells by shaking at 37° for 60 min.	4×10^6 cells
↓	↓	↓
	Shake for 20 min at 37° with 4 ml E. T. Hu. to neutralise unadsorbed virus	
↓	↓	↓
	Pellet cells, discard medium and resuspend in 4 ml E. T. C. and plate.	
↓	↓	↓
	Incubate 24 hrs. 31°C	
↓	↓	↓
	Harvest cells + medium and sonicate.	
↓	↓	↓
	Titrate virus 31° and 38°	

The calculated recombination frequency is corrected for back mutation and leakiness of either parental ts marker by subtracting the correction factor:

$$C.F. = \tfrac{1}{2} (A^{38}/A^{31} + B^{38}/B^{31}).$$

This value was obtained from the control self crosses and is halved for the reason described above, (Methods Section 9a). Therefore the complete formula for calculating recombination frequencies is -

$$R.F.\% = 100 \times 2 \left[(A+B)38/31 - \tfrac{1}{2} (A38/31 + B38/31) \right]$$

(b) Three factor crosses.

The method used was as for two factor crosses but in this case the two ts mutants in each cross differed in plaque morphology i.e. tsAsyn x tsBsyn$^+$. The reciprocal crosses were carried out for each of the possible crosses i.e. tsAsyn x tsBsyn$^+$ and tsAsyn$^+$ x tsBsyn.

11. Heat inactivation.

Virus suspensions were diluted 1:10 in P.B.S.A.C. to give a volume of 2 ml and incubated at 45°C or 55°C. 0.1 ml samples were withdrawn at intervals and assayed for infectious virus.

12. Particle counts.

A sample of the virus stock, diluted in distilled water was

mixed with an equal volume of phospho tungstate acid and an equal volume of a solution of latex spheres of known concentration. A sample of the mixture was examined in the electron microscope. The concentration of virus particles was calculated by comparison of the number of virus particles with the number of latex spheres.

RESULTS

I. General Properties of Wild Type and Mutant Strains of HSV Type 1.

A. Introduction

Before embarking on a genetic analysis of Herpes simplex virus Type 1, it was considered necessary to investigate some of the general properties of the wild type virus, (Glasgow strain 17) and the temperature sensitive and plaque morphology mutants derived from it. This was approached from several directions.

(1) The nature of the plaque morphology and ts mutants.

(a) The nature of the plaque morphology mutant was examined by investigating the structure of the plaques formed in BHK21/C13 cells by syn and syn^+ virus stocks. The stability of the plaque morphology marker at the three temperatures was also investigated.

(b) A comparison was made of the amount of growth displayed by each of the ts mutants at the three temperatures.

(2) Growth patterns in the form of single cycle growth experiments were examined for the wild type virus and each of the ts mutants, to ascertain if there were any gross differences in the time course of virus production. A comparison of the growth cycles of syn and syn^+ mutants was also made.

(3) To determine if any of the ts mutants had alterations in their

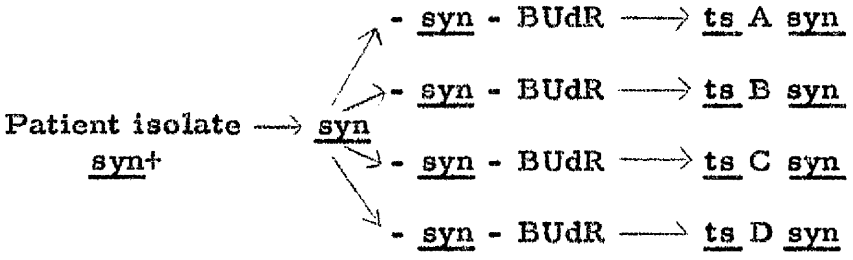
coat proteins which resulted in an altered stability to elevated temperatures, heat inactivation experiments for both wild type and each of the ts mutants were carried out.

(4) Experiments were carried out to determine if breakthrough of the ts mutants at the non-permissive temperature was due to reversion or leakiness.

(5) The particle:p.f.u. ratios of wild type virus and ts mutants and the factors influencing these ratios were investigated.

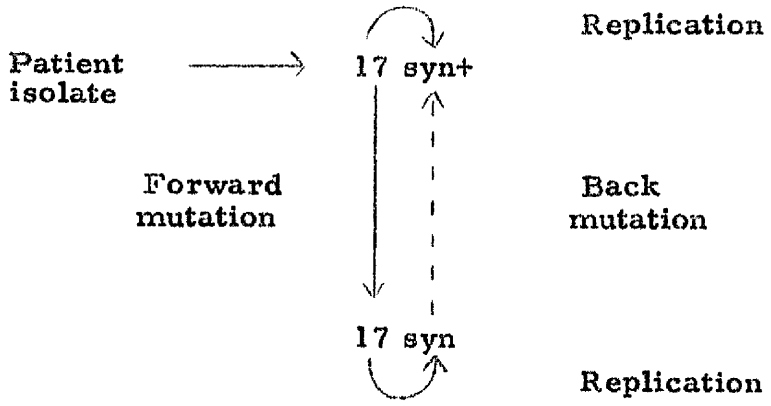
B. Plaque morphology mutant.

The original isolate of HSV gave syn+ plaques in BHK21/C13 cells. A syn plaque arose from the syn+ stock by spontaneous mutation. This was purified by three successive single plaque isolations and a syn stock was grown up. From this, further single syn plaques were grown into separate stocks and these stocks gave rise to one mutant each, after BUdR mutagenesis.



Each of the mutants, as provided by Professor J. H. Subak-Sharpe, was therefore originally available in only the syn form. Since it was apparent that the syn marker was back mutating to syn+ it was

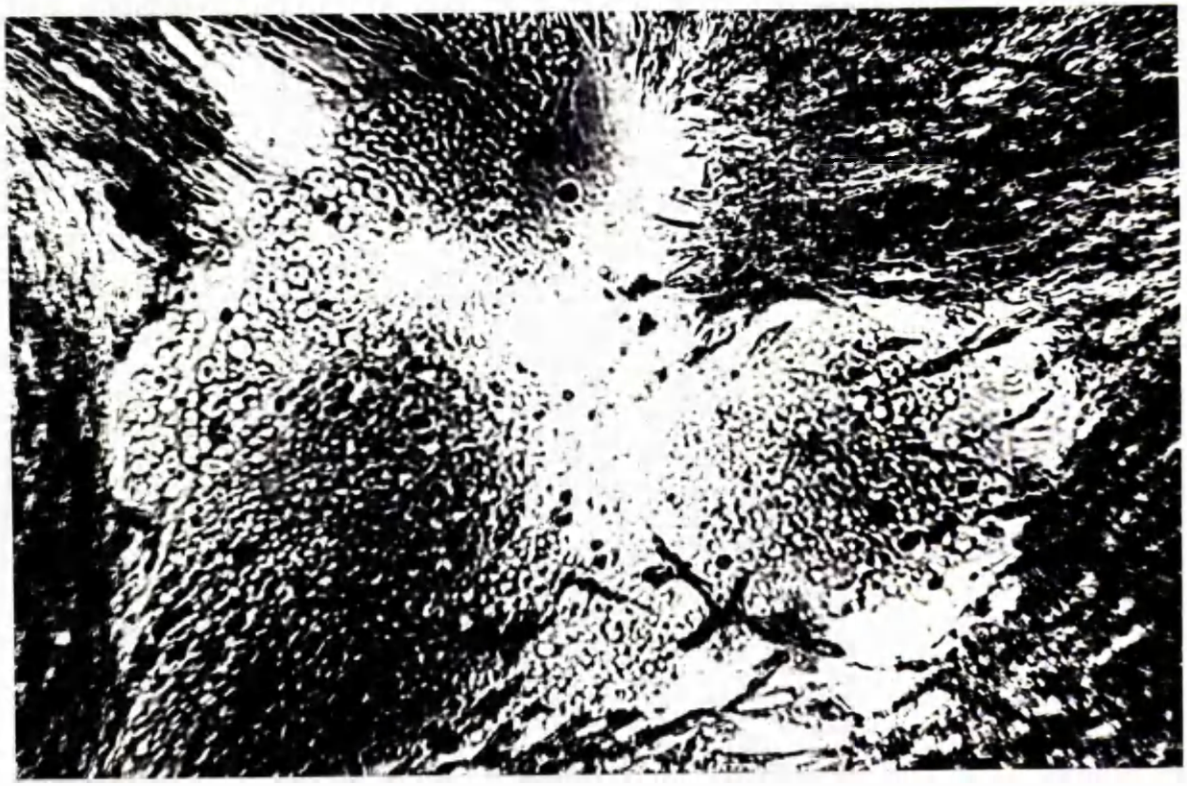
obvious that a complete collection of ts mutants with the syn+ allele could also be obtained. Therefore, I isolated single plaque revertants to syn+ and stocks of each of the ts mutants and the ts+, in combination with either syn or syn+ were obtained.



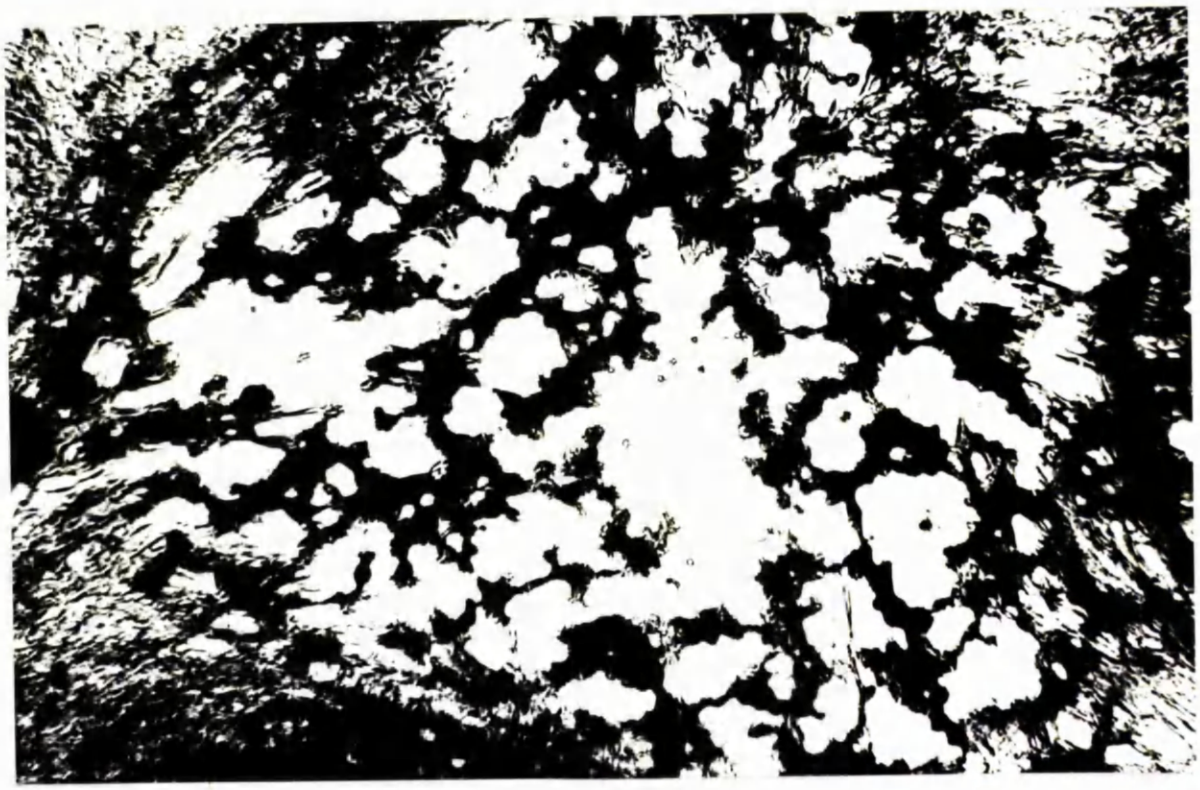
The two types of plaque are quite easily distinguished (Photographs 1a and 1b). The syncytial is formed by the fusion of an infected cell with the surrounding non-infected cells to give rise to giant multinucleate fused cells. The situation is identical to that analysed by Roizman (1962a). The syn+ non-syncytial plaques present quite a different appearance. These are formed of rounded up cells and no syncytia are present. The morphology of both phenotypes remains the same at both the permissive and non-permissive temperatures. The syn+ appears to be slightly dominant over the syn in double infections of the wild type virus i.e. ts+ syn x ts+ syn+.

The average size of plaque reached by 1, 2 and 3 days is 0.25, 0.5 and 1 mm respectively. The syn+ plaques tend on average to be slightly smaller than the syn plaques.

Photograph 1a syncytial plaque syn



1b non-syncytial plaque syn +



C. Temperature sensitive mutants.

Comparative assay of temperature sensitive mutants of HSV at 31°, 36° and 38°C.

Relative titres of two stocks of each ts mutant assayed at 31°, 36° and 38° are shown in Table 4. The titres are those of stocks which gave the lowest and highest observed efficiency of plating (e.o.p.) at the non-permissive temperature of 38°C. The difference in the amount of growth at 38° suggests that all nine mutants (with the possible exception of tsE), revert to wild type, indicating that they are all likely to be single site mutations. The e.o.ps. ranged from less than 10^{-1} to greater than 10^{-7} for the 38°/31° temperatures. For a given mutant the e.o.p. of different stocks at the non-permissive temperature was relatively constant in some cases e.g. tsD, tsE, tsI but for others was more variable e.g. tsA, tsC, tsF and tsG. This variation was shown to be mostly due to 'leakiness' but in addition it could in some cases be shown to be due to back mutation to wild type (Results Section 1F). Where the amount of growth at 38° was due to reversion the efficiency of plating of stocks could be considerably improved by cloning from single plaques. TsF and tsC often gave rise to stocks with high levels of growth at the non-permissive temperature. However it has on occasion been possible to grow stocks with reasonably low background levels. Table 5 shows the efficiency of plating of the stocks which were used in the recombination experiment for the ordering of the

Table 4. Titres of HSV ts mutants at 31°, 36° and 38°C.

ts mutant		p. f. u. /ml 31°	p. f. u. /ml 36°	p. f. u. /ml 38°	Inhibition 36° Log10	Inhibition 38° Log10
A.	(a)	4×10^8	7×10^3	2×10^3	3.8	5.3
	(b)	5.9×10^7	7.4×10^6	4.4×10^4	0.9	3.2
B.	(a)	2×10^9	$<10^2$	$<10^2$	8.7	8.7
	(b)	2.3×10^9	4×10^6	2×10^4	2.8	5.1
C.	(a)	4×10^9	8×10^7	5×10^6	2.7	3.9
	(b)	7.9×10^8	2×10^8	3×10^7	0.6	1.4
D.	(a)	2×10^8	2×10^7	2×10^4	1.0	4.0
	(b)	3.5×10^9	7.4×10^8	2.6×10^6	0.7	3.1
E.	(a)	6×10^8	4×10^6	$<10^2$	2.2	6.0
	(b)	7.9×10^8	8×10^6	4×10^2	2.0	6.3
F.	(a)	3×10^8	10^8	8×10^5	0.5	2.6
	(b)	3×10^8	2×10^8	5×10^7	0.2	0.8
G.	(a)	4×10^7	5×10^4	$<10^2$	2.9	5.0
	(b)	3×10^8	5×10^7	3×10^6	0.8	2.2
I.	(a)	1.5×10^9	1.5×10^7	3×10^5	2.0	3.7
	(b)	2.6×10^8	2×10^5	2×10^5	3.1	3.1
J.	(a)	2×10^8	5×10^4	3.5×10^3	3.6	4.8
	(b)	6×10^8	10^6	3×10^5	2.8	3.3

(a) Lowest observed growth at 38°

(b) Highest observed growth at 38°.

Table 5. Plating efficiency of ts mutants used in the experiment illustrated in Table 21.

Virus		E.O.P. 38°/31°
tsA	(a)	$< 5.0 \times 10^{-6}$
	(b)	$< 4.0 \times 10^{-6}$
tsB	(a)	-
	(b)	$< 1.0 \times 10^{-5}$
tsE	(a)	$< 4.0 \times 10^{-6}$
	(b)	$< 3.5 \times 10^{-5}$
tsC	(a)	1.7×10^{-3}
	(b)	1.5×10^{-4}
tsD	(a)	3.8×10^{-4}
	(b)	$< 4.0 \times 10^{-6}$
tsF	(a)	5.0×10^{-4}
	(b)	1.9×10^{-4}
tsG	(a)	$< 3.0 \times 10^{-6}$
	(b)	$< 5.0 \times 10^{-6}$
tsI	(a)	$< 3.0 \times 10^{-6}$
	(b)	$< 3.0 \times 10^{-6}$
tsJ	(a)	$< 3.6 \times 10^{-6}$
	(b)	-

(a) syn stocks

(b) syn+ stocks

map (Table 21). These stocks were grown from single plaque isolates and it can be seen that in most cases the efficiency of plating was considerably improved, especially for the tsC and tsF stocks. This would suggest a high back mutation rate plus selection.

D. One step growth curves of wild type virus and ts mutants.

(1) One-step growth curve of HSV Glasgow strain 17.

Cell suspensions were inoculated with 5 p. f. u. /cell of ts+syn+ and after adsorption the infected cultures were grown at 31° or 36° or 38°C. Samples were removed at selected times after infection and assayed for total infectious virus (growth medium plus cells) at 31°, 36° and 38°. Figure 1 shows the respective growth curves titrated at 31°C. Titration at 36° and 38° showed similar patterns. The results show that at 36° and 38° infectious virus first appeared about four hours post infection and at 31° new virus first appeared about five hours post infection. Irrespective of temperature virus production continued until twelve hours post infection after which time no significant increase in infectious virus titre was observed, except when the virus was grown at 36° where there seemed to be a slight increase until twenty four hours post infection. The assays at 36° and 38° showed similar increases to twenty four hours post infection. During the growth phase at each temperature a four logs increase in the number of plaque forming units took place. The average yield per cell was found to be about 100 p. f. u.

Figure 1.

One-Step Growth Curve of ts+syn+ Wild Type.

BHK21/C13 cells suspended in E. T. C. were infected at a multiplicity of exposure of 5 p. f. u. /cell with ts+syn+. After adsorption in suspension at 37° for one hour the cells were dispensed in fresh E. T. C. into plastic petri dishes to give 4 x 10^6 cells/dish and incubated at 31°, 36° and 38°C. At selected times post infection duplicate cultures were separately assayed for total infectious virus at 31°C. The values obtained were averaged and the logarithims of p. f. u. /10^6 cells originally plated, plotted against time post infection. Time 0 - after adsorption.

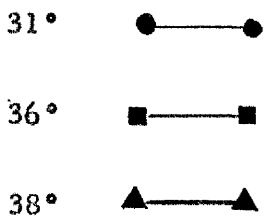
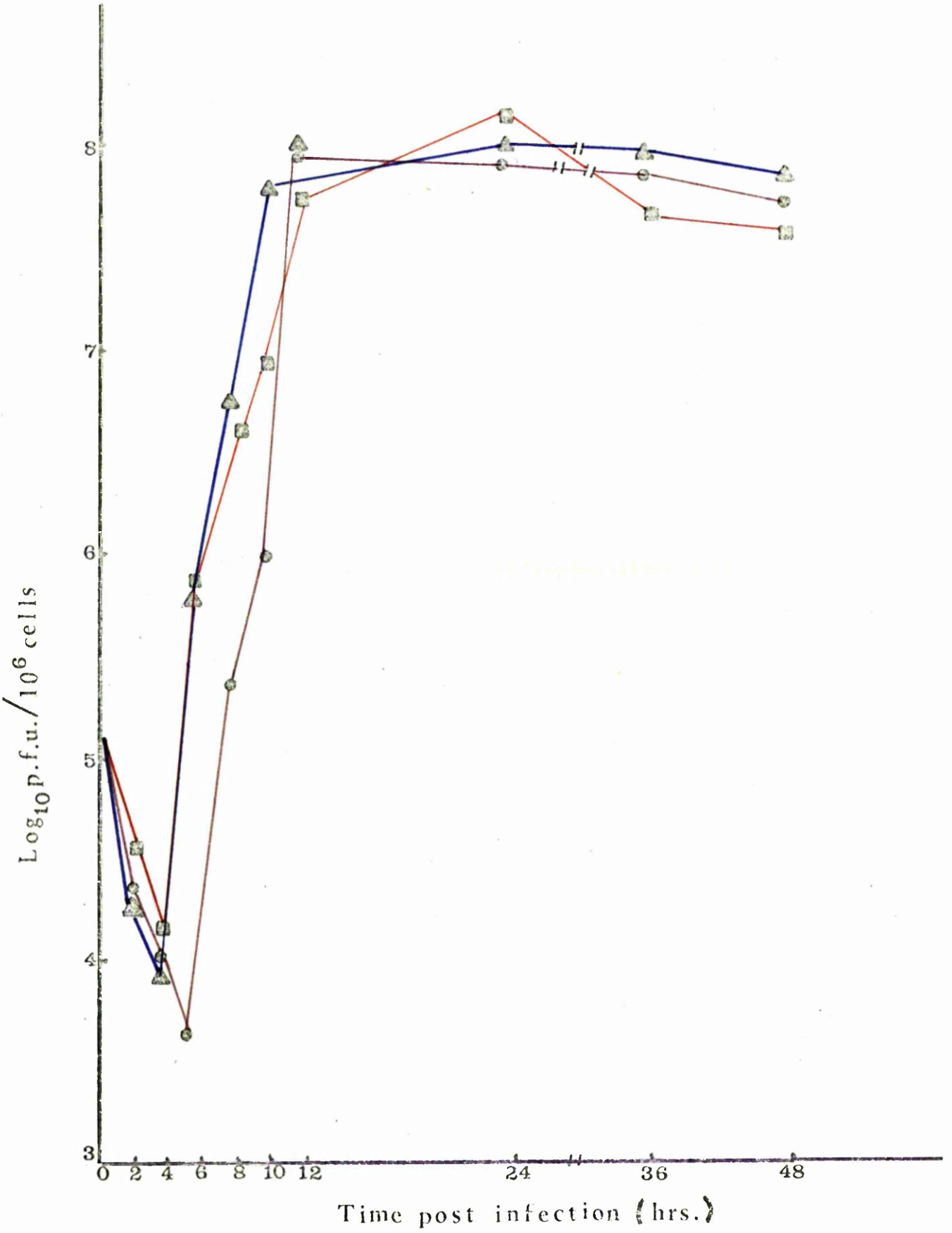


Figure 1


8
7
6
5
4
3
0 2 4 6 8 10 12 24 36 48
Log10 p.f.u./10^6 cells
Time post infection (hrs.)

(2) One-step growth curves of HSV ts mutants.

One-step growth curves were performed under similar conditions but using ts mutant virus. Figure 2a shows the one-step growth curves of tsA and tsB incubated at 31°, 36° and 38° and titrated at 31°C. The results show that for tsA, the first infectious virus was produced at 31° between ten and twelve hours post infection. Virus production continued until twenty hours post infection after which time no significant increase in infectious virus titre was observed. For tsB infectious virus first appeared at 31° between six and eight hours post infection and increased until approximately twenty four hours post infection. For both of these mutants at the permissive temperature, a four logs increase in plaque forming units took place during the growth phase. At 36° tsA had a lag phase similar to that at the permissive temperature but only 1% of the control infectious virus was produced by twenty four hours post infection. For tsB infectious virus first appeared again between six and eight hours post infection and increased until twenty four hours post infection but there was 10% of the control virus produced by twenty four hours post infection. At 38° tsA showed slight growth giving only 1% of the control yield by twenty four hours post infection. TsB on the other hand showed no detectable growth at the non permissive temperature of 38°. The one-step growth curves at 31° of the other ts mutants showed

Figure 2a.

One-Step Growth Curves of HSV Mutants, tsA and tsB.

Experiments were carried out as for Figure 1. Each of the mutants was grown at 31°, 36° and 38° and assayed at 31° for total infectious virus.

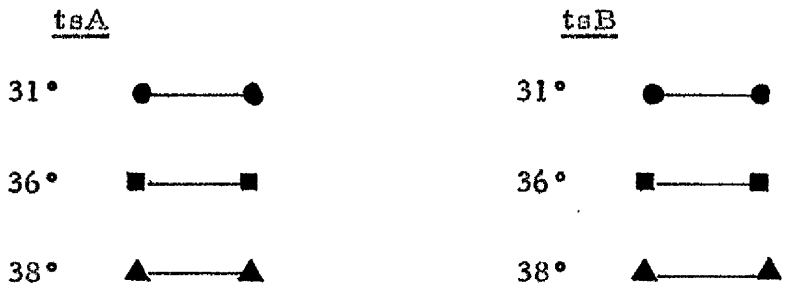
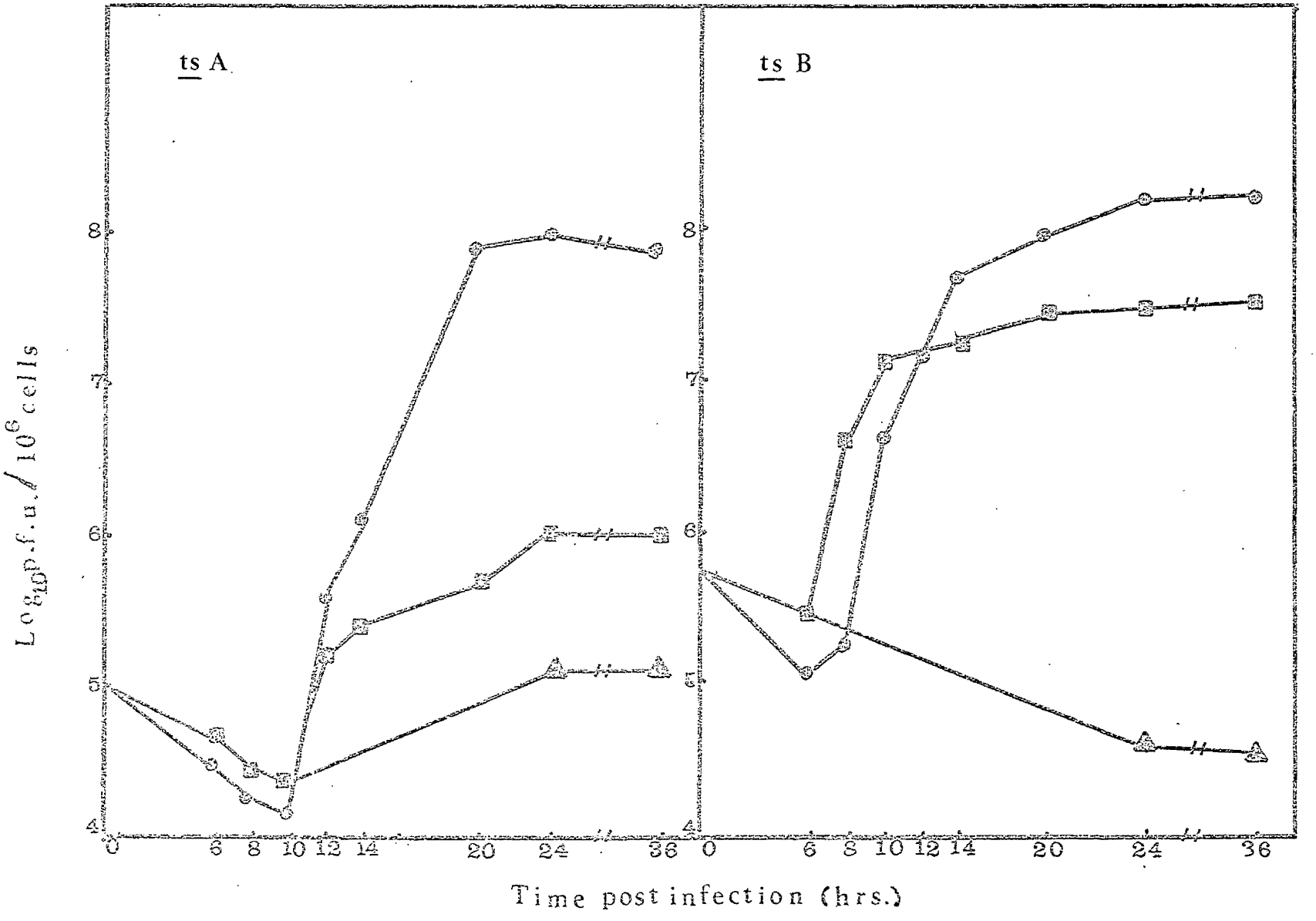


Figure 2a


ts A
ts B
8
7
6
5
4
0
6
8
10
12
14
20
24
36
Log10 p.f.u./10^6 cells
Time post infection (hrs.)

similar patterns (Figure 2b). There were variations in the time of initial production of infectious virus (6-10 hours post infection) and in the time of plateau of the curves (20-24 hours post infection). However the total amount of infectious virus produced by twenty four hours post infection was always approximately the same. There was also variation in the amount of growth of each of the temperature sensitive mutants at 36° (Figure 2c). For each of the mutants their ability to grow at 36° and the level of this growth remained fairly constant from stock to stock. The amount of growth displayed by each of the mutants at 38° is shown in (Figure 2d). The amount of virus growth detectable at 38° varied from stock to stock for some of the mutants but remained constant for others. The reasons for this will be discussed below.

Comparing Figure 1 with Figure 2 it can be seen that at the permissive temperature (31°) the ts mutants grow more slowly than the wild type virus. For the wild type virus the lag phase is four hours compared to between 6-10 hours for the ts mutants. However the increase in infectious virus from the time of initial production of new virus to the time of highest yield was in the region of a ten thousand fold increase in each case. For both the parental strain and each of the ts mutants, the average yield of virus/cell was 100 p.f.u.

Figure 2b.

One-Step Growth Curves of HSV ts Mutants.

Experiments carried out as for Figure 1. Each mutant was grown at 31° and assayed at 31°C for total infectious virus.

tsC

tsD

tsE

tsF

tsG

tsI

tsJ

Figure 2b

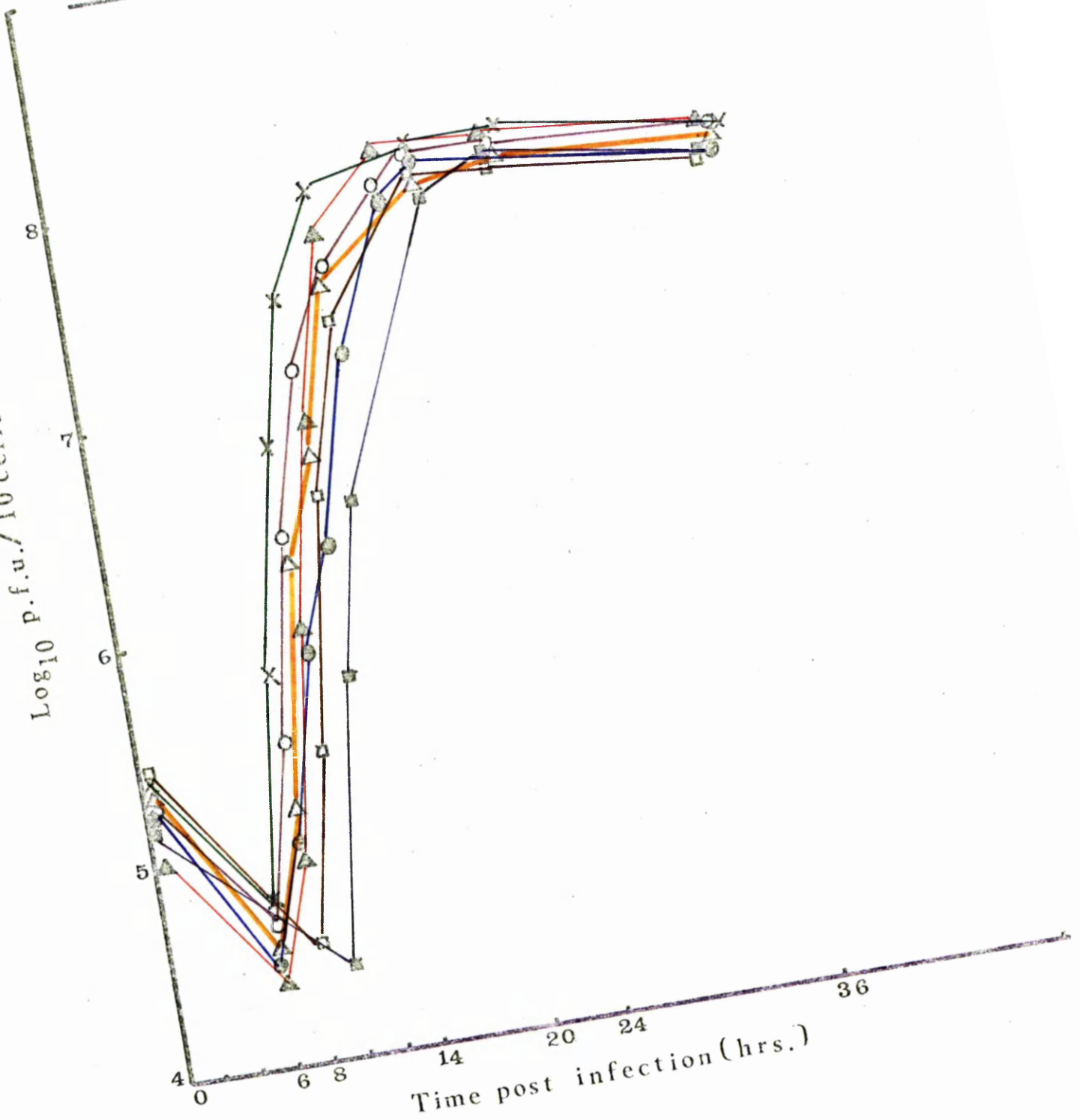

Log10 p.f.u./10^6 cells
8
7
6
5
4
0
6
8
14
20
24
36
Time post infection (hrs.)

Figure 2c.

One-Step Growth Curves of HSV ts Mutants.

Experiments were carried out as for Figure 1. Each mutant was grown at 36° and assayed at 31° for total infectious virus.

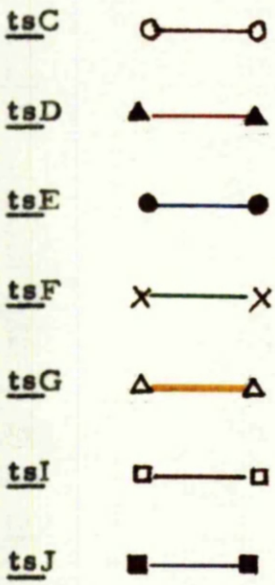


Figure 2c

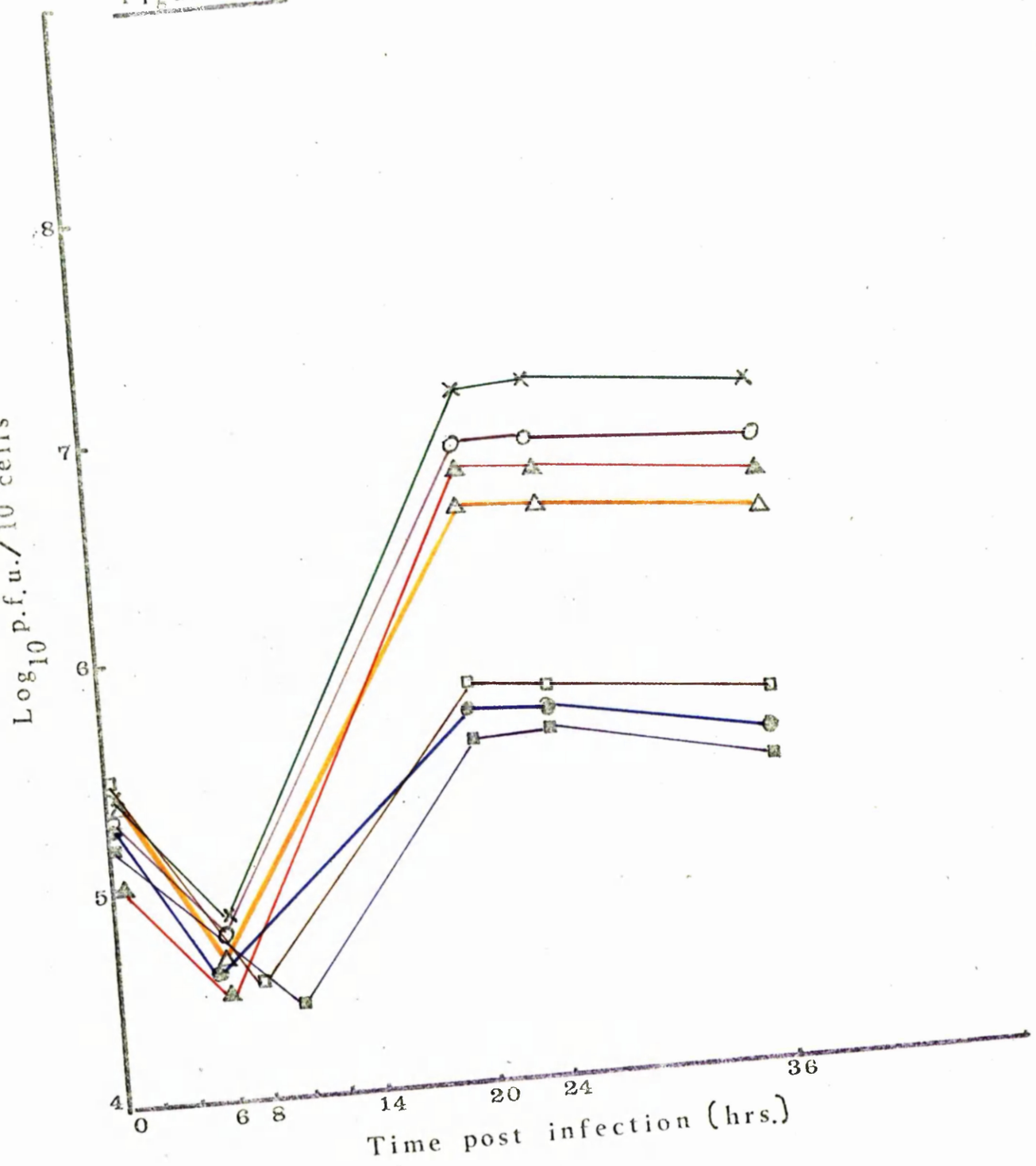

Log10 p.f.u./10^6 cells
8
7
6
5
4
0
6
8
14
20
24
36
Time post infection (hrs.)

Figure 2d.

One-Step Growth Curves of HSV ts Mutants.

Experiments were carried out as for Figure 1. Each mutant was grown at 38° and assayed at 31° for total infectious virus.

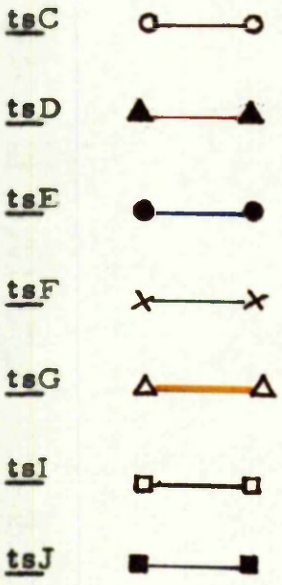
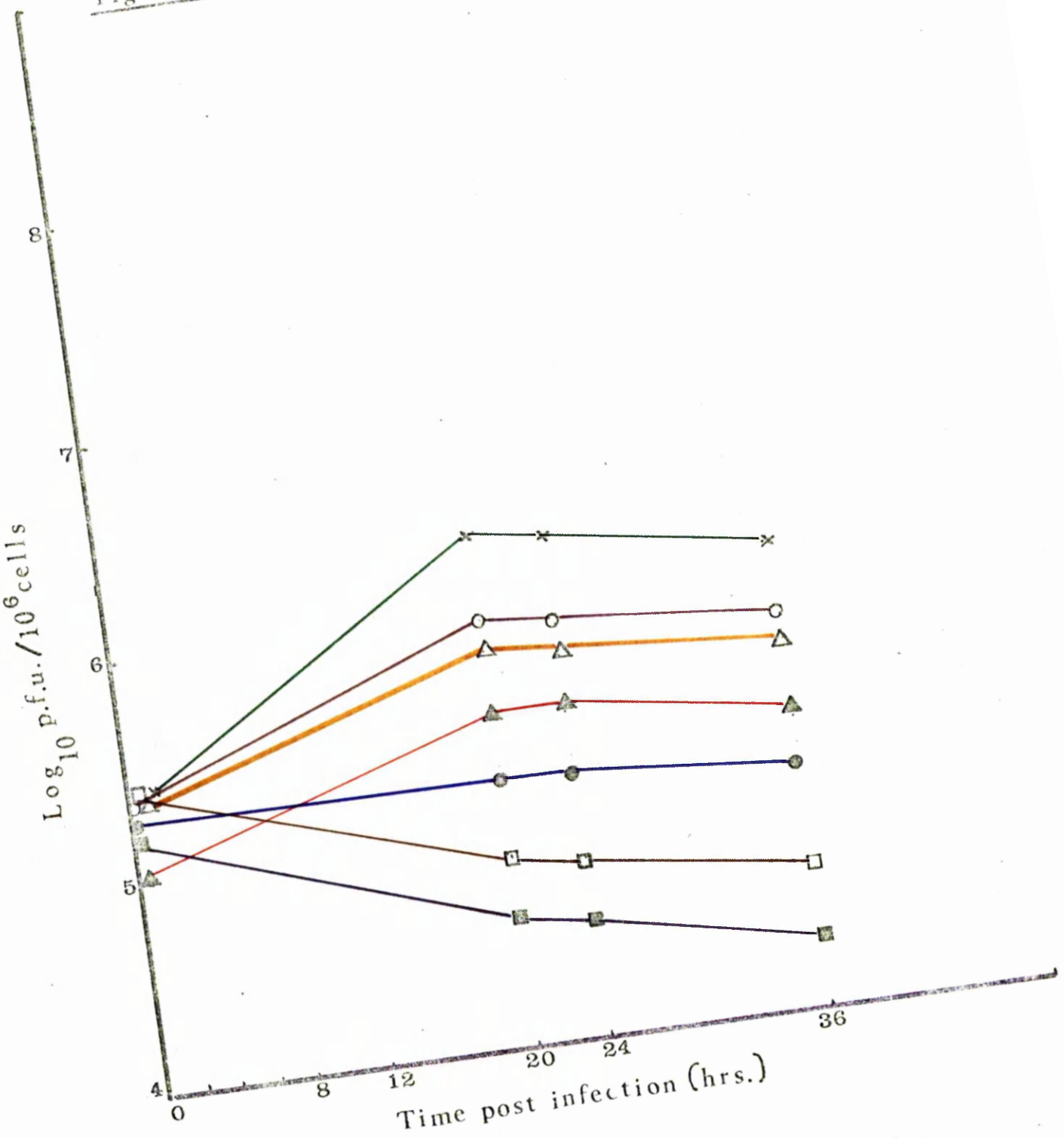


Figure 2d


Log_{10} p.f.u./10^6cells
8
7
6
5
4
0
8
12
20
24
36
Time post infection (hrs.)

(3) One-step growth curves of syn and syn+ ts mutants.

Each of the ts mutants and the ts+ virus is available in two plaque forms syn and syn+ (Results Section 1b). To determine whether the different plaque types affected the patterns of virus growth, single cycle growth experiments were carried out for both. Figure 3a shows the one-step growth curves of tsD syn and tsD syn+ grown at 31°, 36° and 38° and titrated at 31°. Comparing the two growth patterns at 31°, the syn mutant was later in producing new infectious virus, (8 hours for tsD syn; 6 hours for tsD syn+) but the total amount of new virus produced at twenty four hours post infection was greater. For the syn mutant there was a four logs increase in infectious virus between eight and twenty five hours post infection compared to a two logs increase for the syn+ mutant. A similar pattern was observed in the growth curves at 36°. At 38° no infectious virus was produced. The only other mutant for which a similar experiment was carried out was tsG (Figure 3b). Again the syn mutant was later in producing infectious virus and the total increase in new virus was greater.

E. Heat inactivation studies of ts+ and the ts mutants.

Most animal viruses kept in suspension lose infectivity rapidly at room temperature and more rapidly still if they are kept at higher temperatures. At high temperatures heat denaturation of viral

Figure 3a.

One-Step Growth Curves of tsD syn and tsD syn+

Experiments were carried out as for Figure 1. Each of the mutants was grown at 31°, 36° and 38° and assayed at 31° for total infectious virus.

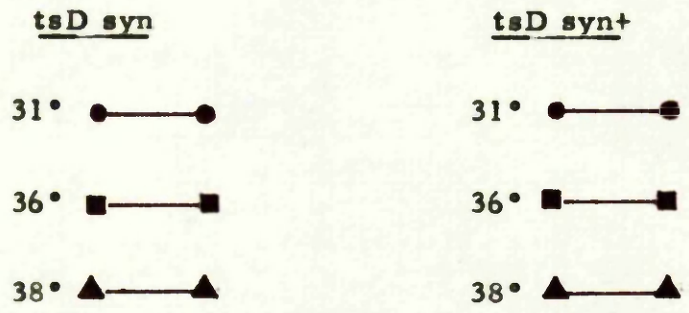


Figure 3a

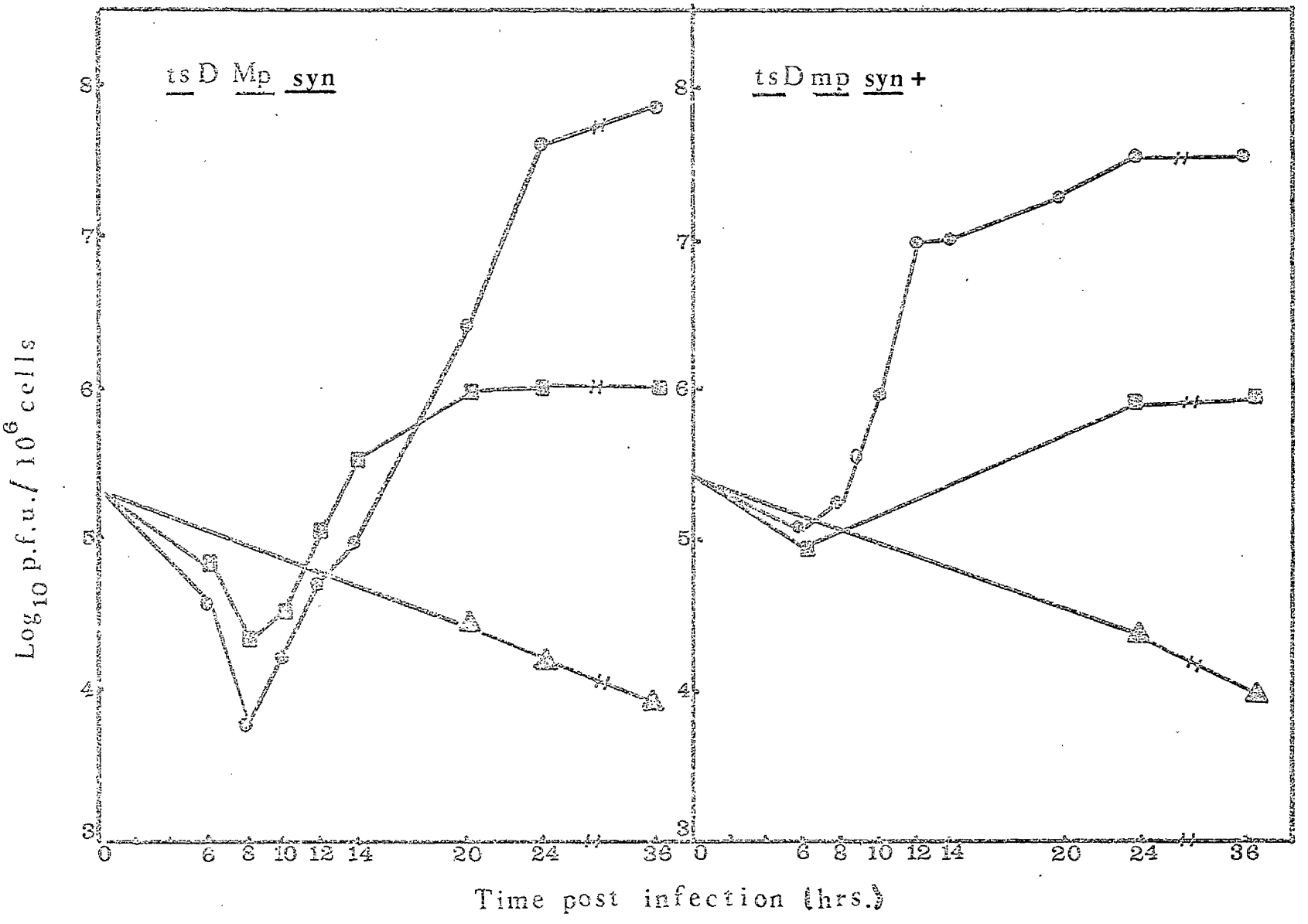

tsD Mp syn
tsD mp syn+
Log10 p.f.u./ 10^6 cells
Time post infection (hrs.)
0
6
8
10
12
14
20
24
36
3
4
5
6
7
8

Figure 3b.

One-Step Growth Curves of tsG syn and tsG syn+

Experiments were carried out as for Figure 1. Each of the mutants was grown at 31°, 36° and 38° and assayed at 31° for total infectious virus.

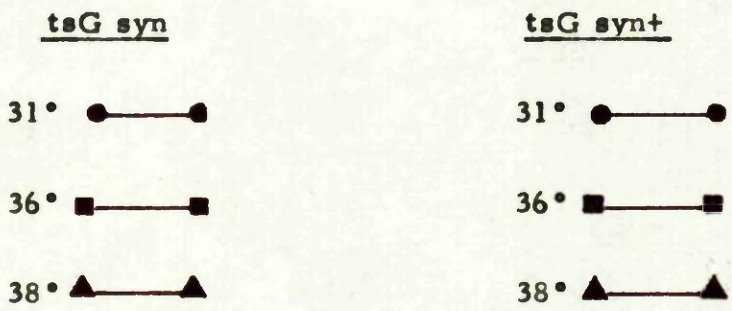
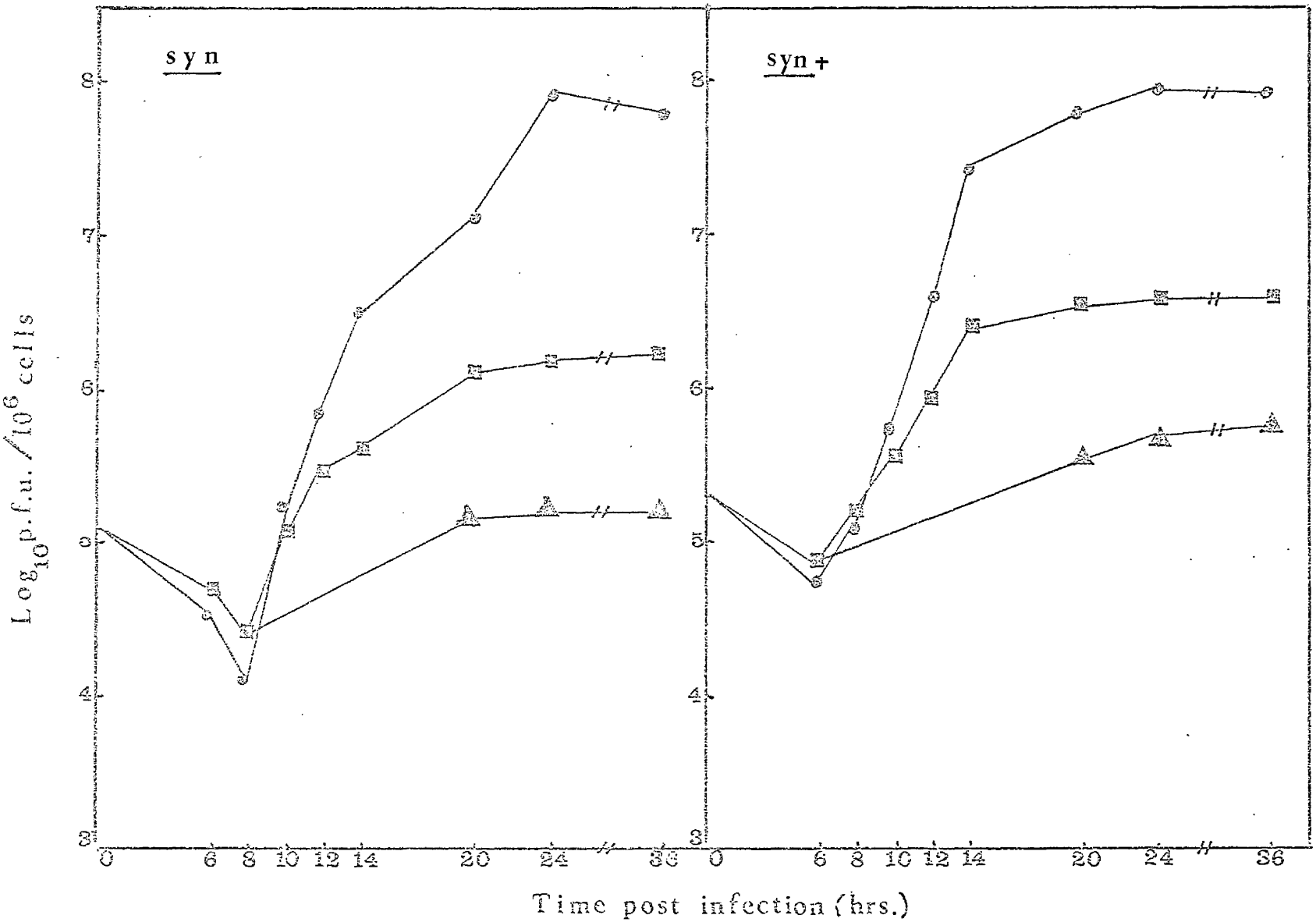


Figure 3b


syn
syn +
8
7
6
5
4
3
0
6
8
10
12
14
20
24
36
Log10 p.f.u. /10^6 cells
Time post infection (hrs.)

protein may lead to disruption of the virion or distortion of the capsid so that normal attachment cannot occur. It is possible that some temperature sensitive mutants have alterations in their coat proteins which render them more susceptible to high temperatures than wild type virus. To determine if this was the case with our ts mutants, heat inactivation experiments were carried out.

The nine temperature sensitive mutants and the wild type virus were diluted 1:10 in P. B. S. A. C. and incubated in stoppered glass bottles in waterbaths at 45°C or 55°. Samples were withdrawn at intervals and assayed for infectious virus by the standard method. All the mutants were inactivated in the same experiment and the samples were taken in order with a 30 second interval between each virus. Figure 4a shows a plot of the surviving fraction against time of incubation at 55° for four of the mutants and the wild type virus. The results indicate that there was a 1. 5 logs decrease in infectious virus after 2. 5 minutes at 55° for the wild type and between 1. 5 and 3 logs for the ts mutants. By five minutes there was between a 3 and 4 logs decrease for both the wild type and ts mutants. The other ts mutants showed similar patterns of inactivation (Figure 4b). The four shown in comparison with the wild type in Figure 4a i. e. tsA, tsB, tsD and tsE were chosen as being representative of the ts mutants in that tsD synthesises DNA, tsA and tsB do not and tsE seems to synthesise a very small amount of DNA (Mechie, et al., 1972). It would be expected that those mutants which make DNA i. e. tsF, tsC,

Figure 4a.

Heat Inactivation of HSV Wild Type and Temperature Sensitive Mutants at 55°C.

Samples of each of the ts mutants and the wild type virus were diluted 1:10 in P. B. S. A. C. to give a final volume of 2 ml and a titre of about 10^8 p. f. u. /ml and immersed in a waterbath at 55°C. Samples of 0. 1 ml were withdrawn at intervals and assayed for infectious virus by the standard method. The log surviving fraction was calculated and plotted against time of incubation.

Glasgow strain 17 ▷——▷

tsA ●——●

tsB ■——■

tsD ▲——▲

tsE o——o

Figure 4a

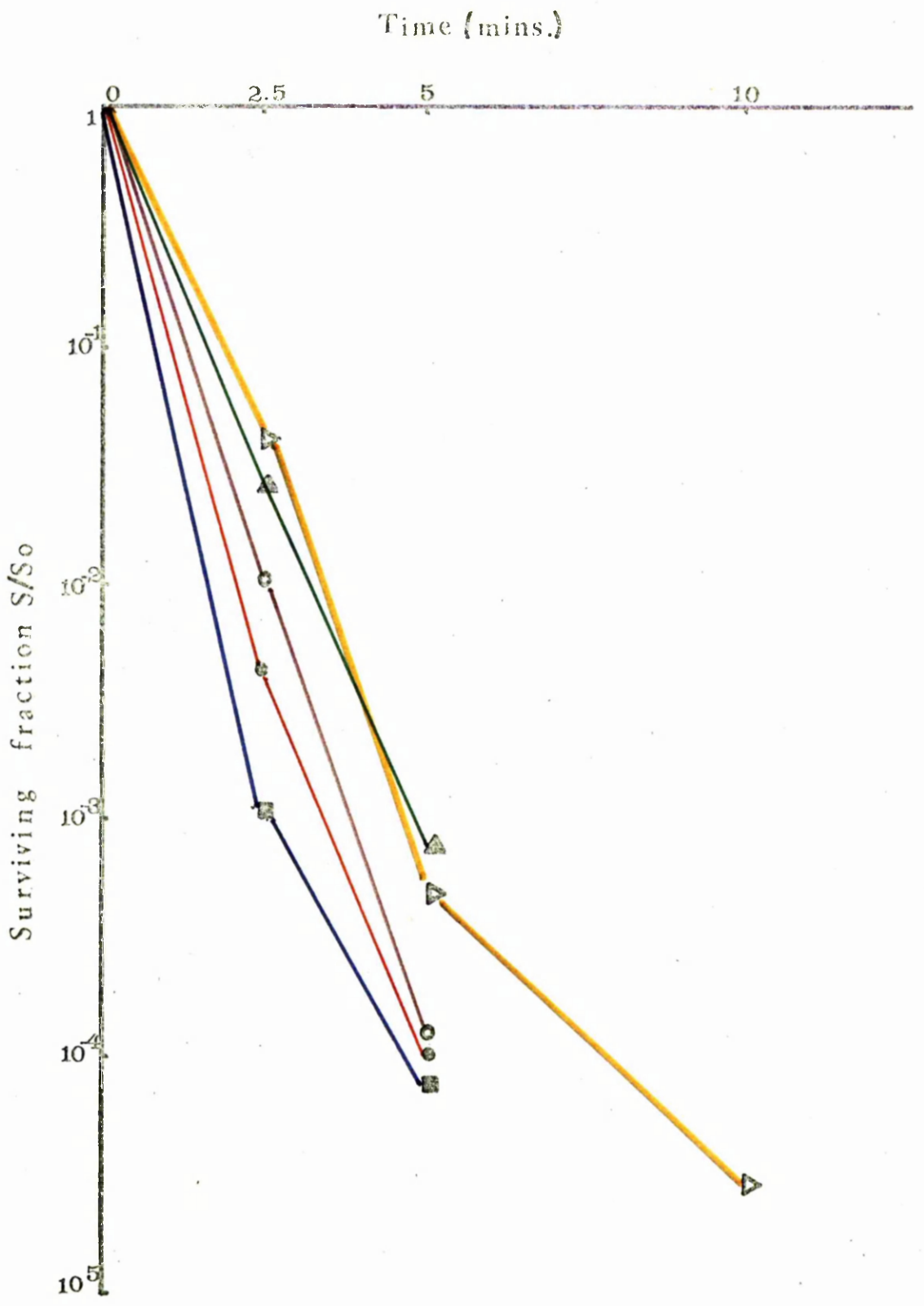

Time (mins.)
0
2.5
5
10
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Surviving fraction S/So

Figure 4b.

Heat Inactivation of HSV ts Mutants at 55°C.

Experiments were carried out as for Figure 4a.

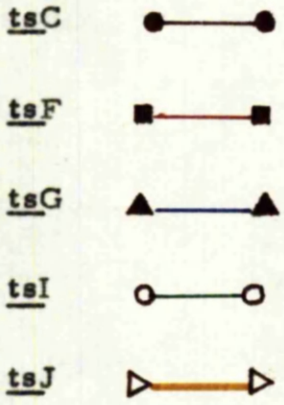
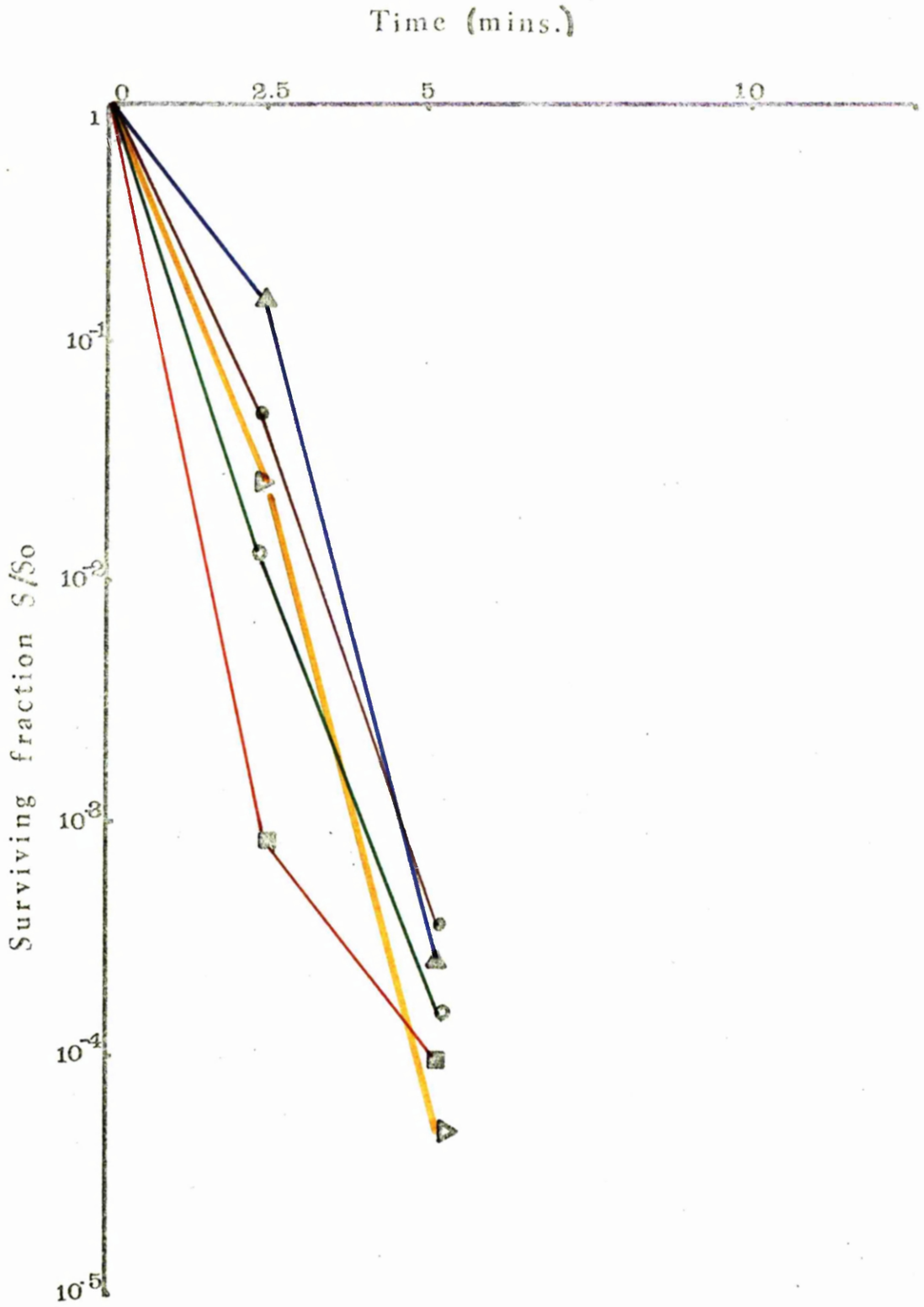


Figure 4b


Time (mins.)
0
2.5
5
10
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Surviving fraction S/So

tsI, tsG and tsD would be the ones which would be most likely to show a difference in inactivation patterns. However the differences in inactivation between any of the mutants and the wild type virus at 55° was not considered as significant.

Because it was considered that five to ten minutes may be too short a time in which to take accurate samples and that a different temperature might reveal differences, the temperature was lowered to 45° and similar experiments repeated. Figure 5a shows a plot of the surviving fraction against time of incubation at 45° for the same four ts mutants and the wild type virus as in (Figure 4a). The results indicate that there was a 1-3 logs drop in infectious virus within one hour at 45° and a 3-4 logs decrease after two hours at 45°. The inactivation curves of the other ts mutants showed similar patterns (Figure 5b). TsE seems to fall off more slowly than the other mutants in the first hour but after this the rate of inactivation was not significantly different.

(Since doing these experiments it has been suggested that 52° may be a more suitable temperature at which to detect differences. It is intended to repeat these experiments at this temperature with more strict controls and to look at the inactivation rates of mixtures of ts+ syn and ts syn+ virus and the reciprocal).

F. Cause of growth of the ts mutants at the non-permissive temperature.

To determine whether growth of the ts mutants at the non-permissive

Figure 5a.

Heat Inactivation of HSV Wild Type and ts Mutants at 45°C.

Samples of each of the ts mutants and the wild type virus were diluted 1:10 in P. B. S. A. C. to give a final volume of 2 mls and immersed in a waterbath at 45°C. Samples were withdrawn at intervals up to 120 minutes after the initial incubation. The samples were assayed for infectious virus by the standard method. The log surviving fraction was calculated and plotted against time of incubation at 45°C.

Glasgow strain 17 ▷——▷

tsA ●——●

tsB ■——■

tsD ▲——▲

tsE o——o

Figure 5a

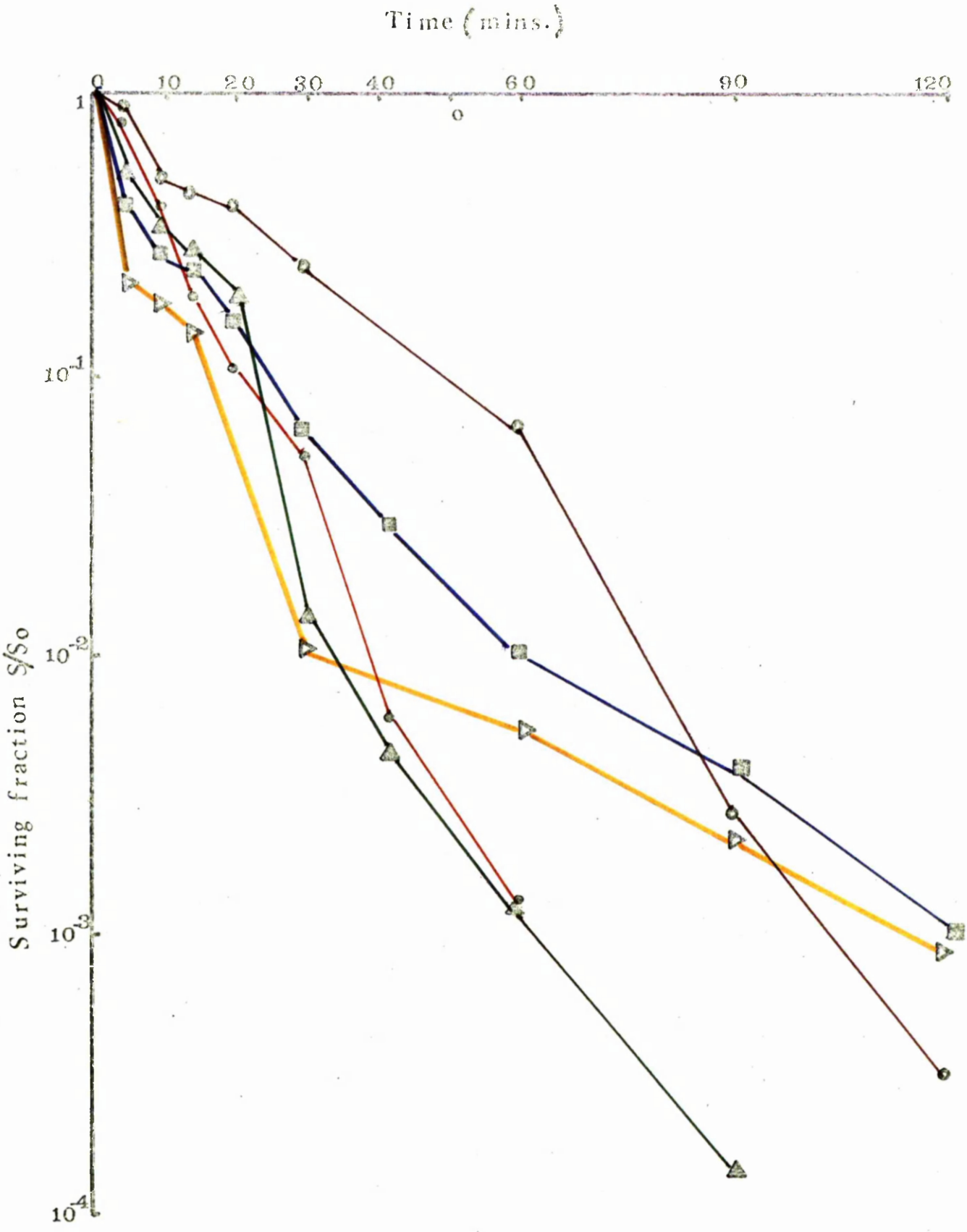

Time (mins.)
0
10
20
30
40
60
90
120
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Surviving fraction S/So

Figure 5b.

Heat Inactivation of HSV ts Mutants at 45°C.

Experiments were carried out as for Figure 5a.

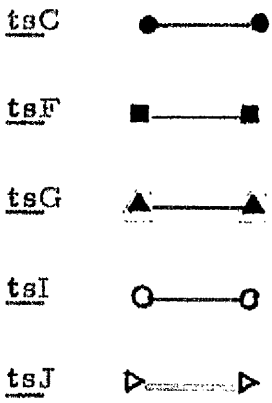
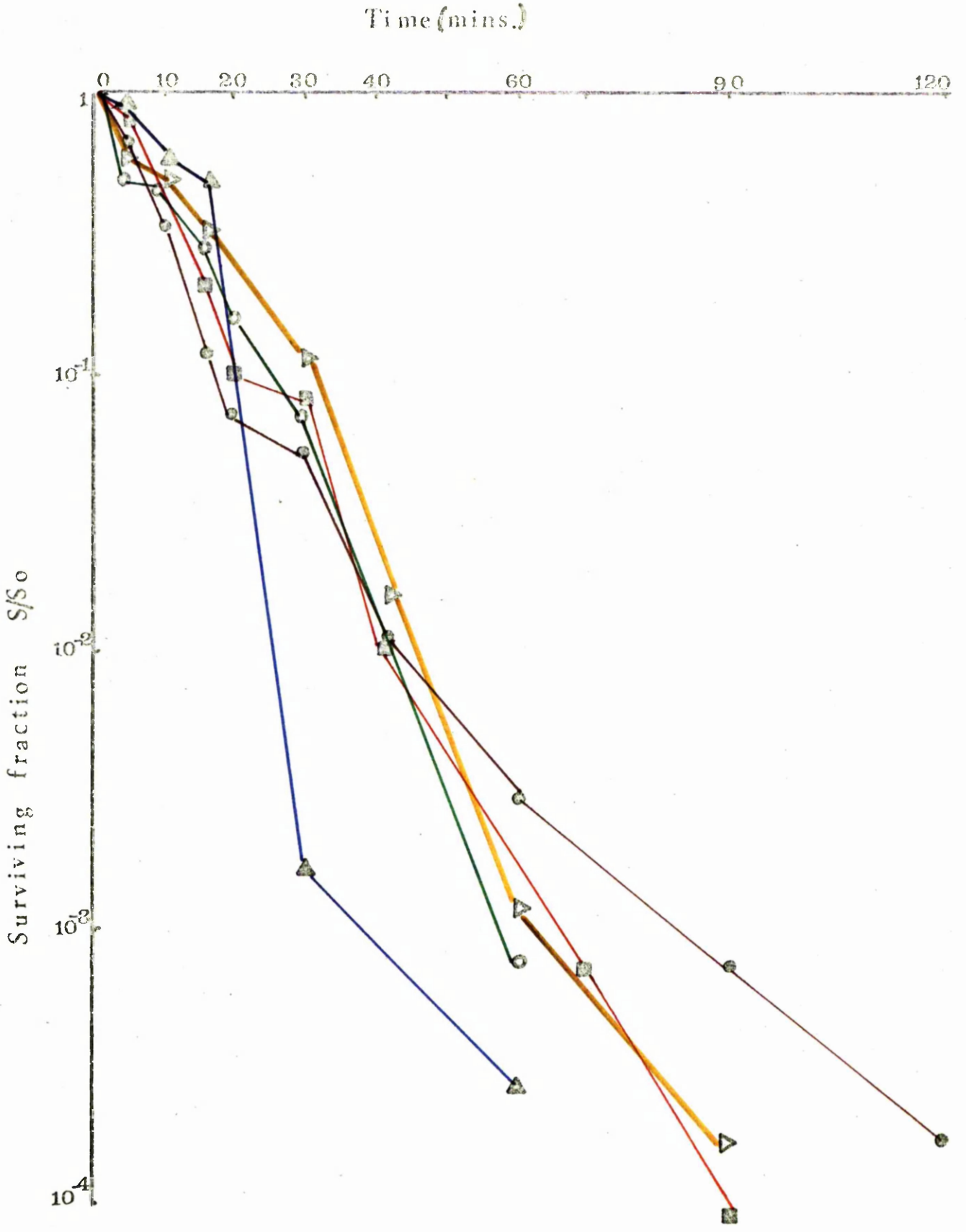


Figure 5b


Time (mins.)
0
10
20
30
40
60
90
120
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Surviving fraction S/So

temperature was due to 'leakiness' (i. e. a mutation which fails to shut off completely the activity of a gene so that some residual expression of its function remains), or to reversion to wild type, single non-overlapping plaques were picked from plates which had been grown at 38°. The plaques were then replated at 31° and 38° and e. o. p. s determined. Revertants would plate equally at 31° and 38°, leaky mutants would still be temperature sensitive. Table 6 shows the number of plaques at each temperature obtained from a single plaque from a 38° plate. The mutants tsC, tsF and tsG were chosen in particular as they showed higher 38°/31° ratios.

The results show that in most cases breakthrough at 38° was not due to reversion of the ts mutants to wild type. If the virus particles picked from the 38° plates had been wild type then similar results as the control ts+ syn+ experiment would have been expected i. e. where virus from a 38° plate gave approximately equal numbers of plaques when regrown at the permissive and non-permissive temperatures. Only in eight cases did the plaques behave as wild type (Table 6, tsF(2) 1, 4 and 8; tsC(2) 4 and 5; tsG 2; tsD 4 and 8). It was concluded that the large amount of breakthrough at the non-permissive temperature observed for the mutants tsF and tsC was in most cases due to leakiness but could on occasion be due to reversion to wild type. (Stocks F (1) and (2) and C (1) and (2) were grown at different times). It should be pointed out that plaques, at the non-permissive temperature which were due to leakiness were in

Table 6. Cause of Growth of the Temperature Sensitive Mutants at the Non-Permissive Temperature.

Ts mutant No. of plaques from 38° plate		No. of plaques 31°C	No. of plaques 38°C	Ts mutant No. of plaques from 38° plate		No. of plaques 31°C	No. of plaques 38°C
A.	1	21	0	F(2)	1	21	16
	2	8	0		2	10	0
	3	11	0		3	18	0
	4	13	0		4	42	36
	5	7	0		5	12	0
	6	19	0		6	17	0
	7	12	0		7	25	0
	8	23	0		8	21	16
C(1)	1	38	0	G.	1	40	0
	2	15	0		2	71	58
	3	95	9		3	2	0
	4	23	0		4	54	1
	5	34	0		5	3	0
	6	127	0		6	20	0
	7	9	0		7	30	0
	8	30	0		8	3	0
C(2)	1	14	0	17+	1	5	0
	2	22	0		2	54	43
	3	18	0		3	31	13
	4	43	40		4	140	137
	5	28	25		5	32	28
	6	8	0		6	0	1
	7	12	0		7	40	32
	8	14	0		8	19	9
D.	1	8	0	F(1)	1	42	0
	2	12	0		2	18	0
	3	15	0		3	0	0
	4	20	16		4	0	0
	5	17	0		5	2	0
	6	5	0		6	6	0
	7	8	0		7	5	0
	8	14	12		8	3	0

general much smaller in size than the corresponding plaques at 31°. At the same time these results give good evidence that reversion does occur with tsC, tsD, tsF and tsG.

G. Comparison of particle and infectivity counts of HSV stocks.

Standard plaque assays and particle counts of virus stocks were carried out according to the method described (Methods Section 12). Table 7 shows a comparison of the particle:p. f. u. ratio of two stocks of ts+ syn+ which had been grown on separate occasions. The degree of variability in the particle:p. f. u. ratio was observed in virus stocks grown at different times. As the multiplicity of infecting virus was always constant (1:300), the variability was most likely to be caused by the condition of the cells and/or the particle:p. f. u. ratio of the seed virus. If the seed virus contains a high proportion of non-infectious particles this may have an effect in blocking the adsorbing ability of the cells or the ability of the cells to produce virus. As the stocks are grown from low multiplicity infections over three cycles, production of high titre stocks depends to a large extent on the ability of cells to adsorb virus from first and second cycles.

Table 8 shows a comparison of the particle:p. f. u. ratios of wild type and ts mutants and the effect of empty particles on the particle:p. f. u. ratio. The results indicate that there was little difference between the particle:p. f. u. ratios of wild type and ts mutants. This was true for all the ts mutants. It can be seen also

Table 7. Particle:p.f.u. Ratio of ts+ syn+

p.f.u./ml	particles/ml	p.f.u.:particles
1.28×10^{11}	4×10^{11}	1:4
1.6×10^{8}	4.4×10^{9}	1:30

Table 8. A Comparison of Particle:p. f. u. Ratios of Wild Type and ts Mutants of HSV and the Effect of Empty Particles on the Particle:p. f. u. Ratios.

Virus	p. f. u. /ml	part. /ml	%empty part.	full part/ml	p. f. u. : part.	p. f. u. : full part.
ts+ syn	10^8	3.6×10^9	70	1.1×10^9	1:36	1:11
tsD	1.5×10^8	6×10^9	85	9×10^8	1:40	1:6.6
tsG	1.5×10^8	6.6×10^9	72	1.9×10^9	1:44	1:13
tsA	3.4×10^8	9.2×10^9	40	5.5×10^9	1:27	1:17
tsJ	2.4×10^8	5.6×10^9	52	2.7×10^9	1:23	1:11

Table 8a. Effect of the Source of the Virus on the Ratio of Enveloped to Naked Particles and the Particle:p.f.u. Ratio.

(a) Spontaneously released extracellular Glasgow strain 17.

p.f.u./ml	particles/ml	% enveloped particles	particles:p.f.u.
9×10^9	4.8×10^{10}	40	1:5
3×10^9	4×10^{10}	30	1:13

(b) Virus released from cells by sonication Glasgow strain 17.

p.f.u./ml	particles/ml	% enveloped particles	particles:p.f.u.
2×10^9	8.19×10^{10}	<a	1:40
2.5×10^9	1.89×10^{11}	<a	1:75

that the proportion of empty particles in a stock had a considerable effect on the ratio. Some of these stocks had particularly high proportions of empty particles. In general the percentage of empty particles in a stock is fairly low. The reasons for large amounts of empty particles being produced are not fully understood but to some extent it appears to depend on the batch of cells being used for virus growth. It should be pointed out however, that what would appear to be empty particles in negative stain preparations as seen in the electron microscope may not be true empty particles but artefacts produced by the stain.

Table 8a shows the effect of the source of the virus on the ratio of enveloped to naked particles and the particle:p. f. u. ratio in a stock. The results indicate that the source of the virus has a considerable effect on the proportion of enveloped particles in the population and this in turn has an effect on the particle:p. f. u. ratio. In general spontaneously released virus contains more enveloped particles than intracellular virus. This is probably a reflection on the source of the envelope. The origin of the envelope and its role in the infectious process is discussed in the general introduction. However it would appear from our results that the envelope has an effect on the infectivity of the particle. The above two samples of artificially released virus had no visible enveloped particles in the sample examined. This was unusual in that most preparations of intracellular virus examined had a small proportion of enveloped particles. In

our experiments we tried to use virus with a particle:p. f. u. ratio of approximately as good as 1:10.

(It should be pointed out that all samples examined in the electron microscope were from fresh stocks and not from stocks stored for any time at -70°C).

H. Discussion.

As a prerequisite for the genetic studies to be described later a ts syn+ revertant was isolated for each ts syn mutant. It has been shown that the two plaque morphology variants (syn and syn+) of Glasgow strain 17 are easily distinguishable and are stable at both the permissive and non-permissive temperatures. One variant, syn+, forms rounded cell plaques in BHK21/C13 cells and these plaques ultimately dislodge from the surface of plates. When there is no neutralising antibody present in the overlay medium the syn+ plaques spread to give comet-shaped plaques and small satellite plaques. These are formed by the virus being released from the rounded cells and infecting the surrounding cells. The other variant, syn, forms giant multinucleate fused cell plaques in the presence or absence of neutralising antibody. Virus is passed from infected cell to surrounding cells by fusion and it is not necessary for virus to be released into the medium before infecting surrounding cells. These two effects on cell structure have been well documented (Roizman, 1962). In 1968 Herpes strains were in fact divided into four groups

depending on their differing effects on the social behaviour of infected cells (Ejercito et al., 1968). The four classes were:- (1) Strains causing rounding of cells but no adhesion or fusion. (2) Strains causing very tight adhesion of rounded cells. (3) Strains causing loose aggregation and rounded cells and (4) Strains causing polykaryocytosis. On the basis of this classification our syn+ mutant would fall into category (1) and the syn mutant into category (4). The fact that Glasgow strain 17 gives both categories of plaques and their relationship is a single mutational step throws great doubt on the usefulness of Ejercito et al.'s classification.

It has been shown that each of the ts mutants reverts to wild type (Tables 4 and 6). This was taken as being indicative that the mutants were single step mutants and were not double or multiple mutants. If they had been doubles then it would be expected that reversion to wild type would have been lower than observed. Since multiple mutants can cause considerable confusion in genetic mapping it was considered advantageous that each of the ts mutants appeared to be a single step mutant. On the other hand with single step mutants revertants tend to accumulate more frequently but this problem was overcome by regular plaque purification and the growing of stocks from single plaques, provided that the wild type had a selective advantage. One problem which could not be overcome was the relatively high leak rate of tsF and tsC. With these two mutants, when the amount of growth at 38° was due both to leakiness and reversion the situation

could be slightly improved by cloning but was not at any time completely overcome. It has been found with other viruses that ts mutants tend to be leaky but they rarely function as effectively as wild type virus.

Similar patterns for one-step growth cycles of HSV Type 1 have been reported by Roizman et al., (1963) using the macro plaque strain Mp HEp 2 cells. They found that at 34° new virus first appeared 5-6 hrs after infection; multiplication followed at exponential rate until 13-15 hrs after infection and by 6 and 13-15 hrs., the titre of virus doubled every forty minutes. After 17-18 hrs post infection the titre remained constant or decreased. Levitt and Becker (1967), found, using the HF strain of virus and BSC1 cells that infectious virus began to accumulate in infected cells 6 hrs post infection and reached high titre at 18 hrs post infection at 37°. Klemperer et al., (1967) following the growth of the HFEM virus strain in BHK21/C13 cells found that new virus particles were first produced 3 hrs after infection and increased to 17 hrs post infection at 37°. In general it has been shown that the duration of the eclipse ranges from 3-8 hrs and is affected by the temperature (Farnham and Newton, 1959; Hoggan and Roizman, 1959; Smith, 1963). The differences observed in our growth patterns at 31° and 38° would confirm this. It has also been shown to be affected by the m.o.i. and prior infection by another mutant (Roizman, 1963; 1965). The duration of the cycle is also known to be affected by the m.o.i.

and the temperature of incubation (Roizman, 1963), and we have shown in our results that the growth cycle of the wild type virus at 31° is slightly slower than at 38°. The virus yield has been shown to increase exponentially from the end of the eclipse to almost the end of the reproductive cycle. Under optimal conditions in HEp 2 cells the virus yield has been shown to be between 10,000 to 100,000 virions/cell (Roizman, 1963). The estimate of 10^5 virions/cell seems rather large when the molecular weights of the viral and cell DNAs are considered. The M. W. of the virus D.N.A. is 10^8 daltons, that of the cell DNA is 10^{12}. Therefore for the cell to make 10^5 virions it would have to make approximately 10 times its total DNA. With Glasgow strain 17 the average yield/cell is about 100 p. f. u. /cell. Therefore it would appear that although there are variabilities due to virus strain and cell line, as well as experimental conditions, the patterns reported for one-step growth curves of HSV Type 1 fit well to the growth curves we have shown for Glasgow strain 17. One interesting feature of the growth patterns of the ts mutants is that they are invariably slower than those of the wild type virus. This has also been demonstrated with the ts mutants of HSV Type 2 (Halliburton, 1972). It has been suggested that this may be due to mutations at sites other than the ts and that by studying the growth cycles of revertants from ts to wild type we will be able to ascertain if this is the case.

It can be seen from the results that the p. f. u. :particle ratios varied from one preparation to another and that the ratios appeared to

depend on the proportion of enveloped particles in the preparation. The proportion of enveloped particles tended in turn to depend on the source of the virus i. e. whether it was spontaneously released or artificially released. This of course reverts to the age-old problem of the role of the envelope in the infectious process, which was discussed in the general introduction. However our preparations had in general a particle:p. f. u. ratio of approximately 10:1.

From the preliminary heat inactivation studies, it would appear that none of the ts mutants is more heat stable or heat labile than any other or the wild type virus. It should be pointed out however that as it is difficult to do inactivation studies in a medium free from compounds protecting or competing with the virus for the inactivating agent, conclusions that one virus is more or less stable than another may well be unfounded. However as this does not apply so much to heat and as we were only dealing with one strain of virus, although different mutants of that strain, we would feel justified on the basis of our results to date, to conclude that each of the ts mutants and the wild type virus are inactivated at the same rate.

II. Genetic Analysis of Temperature Sensitive Mutants of HSV by Complementation Tests.

A. Introduction

The nine temperature sensitive mutants were studied for their ability to complement in mixed infections at the non-permissive temperature in order to identify the number of functional groups so far involved. If complementation could be shown to occur with reasonable efficiency, this study would form the basis for more extensive studies on the genetic content and structure of the HSV genome. All possible pairwise combinations were carried out. The complementation analysis was approached from three directions:-

(1) Infectious centre assay.

Assay of the number of infectious centres produced at the non-permissive temperature in mixed infections compared with the parents alone. If the two ts mutants in a cell are complementing each other, then the infected cell should produce an infectious centre plaque at the non-permissive temperature. Cells infected with only one mutant or non-complementing mutants will not produce plaques at the restrictive temperature.

(2) Yield experiment.

Assay of the yield of progeny virus from mixed infections and single parent infections grown at the non-permissive temperature. If the two ts mutants are complementing each other then the yield from mixedly infected cells at the restrictive temperature should

be significantly increased over the yields from cells infected with non-complementing mutants or single mutants.

(3) Plaque morphology analysis.

Observation of the plaque morphology of infectious centres of mixed infections at 38° in which one parent (ts mutant) was syn and the other syn+. With complementing mutants the infectious centre plaque would be expected to exhibit mixed syn/syn+ morphology. In mixed infections of ts+ syn+ and ts+ syn, the syn+ mutant was shown to be slightly dominant over the syn mutant.

B. Infectious centre test.

Initially the complementation analysis was performed by measuring the frequency of infectious centres produced at the non-permissive temperatures of 36° and 38°C.

The results of one experiment in which all infections were carried out in the same experiment are given in Tables 9 and 10. Table 9 gives the relative plaque titres and these were used to calculate the complementation indices presented in Table 10 (Methods Section 9a). In most cases the infectious centre titre of the mixed infections exceeded those of the controls and this was taken as evidence of positive complementation. At 38° the indices varied from about 1.0 (no evidence of complementation) e.g. tsA x tsE, tsA x tsJ and

Table 9.

Infectious Centre Assay (1)

4×10^6 BHK21/C13 cells were infected at a m.o.i. of 5 p.f.u./cell of each of the two ts mutants. After adsorption and neutralisation of residual virus the infected cells were added to control cells and the plates incubated for 2-3 days at 31°, 36° and 38°C. The number of plaques i.e. infectious centres were counted after fixing and staining.

(1) Titre of infectious centre at 36°.

(2) Titre of infectious centre at 38°.

(3) Titre of infectious centre at 31°.

(The 38° figure is the average from two plates)

ts Mutant		A	B	C	D	E	F	G	I	J
A	(1)	3×10^2	5.6×10^3	3.2×10^3	3.7×10^3	2.2×10^3	2.7×10^4	1.1×10^4	3.5×10^3	2.8×10^3
	(2)	<10	<10	2.5×10^3	5.1×10^2	<10	5.9×10^3	6.8×10^3	6.7×10^3	<10
	(3)	2.5×10^4	2.9×10^4	3×10^4	3×10^4	3.3×10^4	3.6×10^4	4.5×10^4	3.7×10^4	4.5×10^4
B			5×10^2	2.7×10^4	2×10^3	3×10^2	1.2×10^4	2×10^4	8.2×10^3	1.9×10^4
			<10	1.3×10^3	8.3×10^2	<10	7.2×10^3	1.1×10^4	2.4×10^3	3.3×10^3
			2.5×10^4	3.5×10^4	3.5×10^4	5.2×10^4	1.5×10^4	7×10^4	5×10^4	7×10^4
C				2.2×10^3	2.5×10^3	1.1×10^3	2.3×10^4	2.7×10^4	4×10^4	1.8×10^4
				5×10^2	2.8×10^3	1.5×10^3	9.4×10^3	1.7×10^4	6×10^3	2.8×10^3
				3×10^4	4.3×10^4	4×10^4	4×10^4	10^5	9.2×10^4	5.6×10^4
D					9×10^2	9×10^2	4×10^4	1.6×10^4	2.7×10^2	9×10^2
					9×10^1	2.5×10^3	2.7×10^3	1.4×10^4	1.7×10^2	6×10^1
					9.3×10^4	3.2×10^4	2.7×10^4	10^5	3.6×10^4	7×10^4
E						5×10^1	5×10^4	5×10^4	6.2×10^2	7.7×10^3
						10	8×10^3	1.9×10^3	2×10^2	4.5×10^2
						8.6×10^4	1.1×10^6	10^5	4×10^4	7×10^4
F							2.3×10^4	2.8×10^4	4.4×10^4	7×10^4
							1.6×10^3	1.1×10^4	2.3×10^3	4.2×10^3
							3×10^4	7.2×10^4	6×10^4	3.6×10^4
G								2.7×10^4	1.4×10^4	7.3×10^3
								3.1×10^3	3.9×10^3	1.5×10^3
								8×10^4	10^5	3.7×10^4
I									6×10^2	6×10^3
									10	5.1×10^2
									7.6×10^4	6.4×10^4
J										<10
										<10
										8×10^4

tsB x tsE to greater than 100 e.g. tsA x tsI, tsB x tsI and tsB x tsJ. Mutants tsF and tsC were rather leaky - and as a consequence infections involving either mutant tended to show low complementation indices. The C.I. 36/31 were often lower than the C.I. 38/31. Taking a C.I. of 2 or greater as evidence of positive complementation four pairs failed to show positive complementation at 38° but did show it at 36° viz. tsA x tsE, tsA x tsJ, tsD x tsJ and tsE x tsG. Only three pairs failed to show complementation at either temperature viz. tsB x tsE, tsF x tsI and tsG x tsI. Breakthrough at the non-permissive temperature which was established as being due to leakiness (Results Section 1F) was taken into consideration when analysing the results, and corrections were made where necessary. For a given pair of mutants the complementation index often varied in successive experiments. Table 11 shows the complementation indices calculated from the results of an infectious centre assay carried out at a different time with different virus stocks. All the infections were carried out in the same experiment. The results showed considerable variation from those presented in Table 10. There was a much higher proportion of combinations showing no detectable complementation at 36° or 38°. The degree of variability in successive experiments is also illustrated by the complementation indices calculated for selected crosses from five separate experiments e.g. at 38° tsE x tsI gave indices of 5.0, 29, 43, 206 and 428; tsI x tsJ gave indices of 1.7, 5.0, 46, 70 and 80. In other cases the values were more consistent,

Table 10.

Infectious Centre Assay (1)

Complementation coefficients calculated from the results illustrated in Table 9 (Methods Section 9a).

(a) 36° C.I.

(b) 38° C.I.

Table 10. Complementation Indices from Infectious Centre Assay (1)

ts Mutant		A	B	C	D	E	F	G	I	J
A	(a)	1	17.8	2.8	22.4	74.1	2.0	1.5	93.1	>88.9
	(b)		<8.5	9.5	24.3	<1.5	6.1	5.9	>595	<0.7
B			1	16.4	3.9	0.6	2.0	1.6	15.9	>25.9
				4.2	34.0	<0.7	17.9	8.0	>163.3	>157
C				1	13.6	7.4	1.4	1.2	12.5	>8.8
					7.4	4.5	6.7	2.4	7.8	5.9
D					1	5.4	3.8	0.9	1.4	>2.6
						131.0	3.6	6.8	8.0	1.5
E						1	1.2	3.0	22.1	>275
							2.7	1.0	5.0	64.0
F							1	0.7	1.9	5.1
								3.3	1.4	4.4
G								1	0.8	12
									1.5	2.1
I									1	187.6
										80.0
J										1

Table 11.

Complementation Indices from Infectious Centre Assay (2)

Complementation indices calculated from a separate infectious centre assay (Methods Section 9a).

(a) 36° C.I.

(b) 38° C.I.

Table 11. Complementation Indices from Infectious Centre Assay (2)

ts Mutant		A	B	C	D	E	F	G	I	J
A	(a)	1	>0.5	>0.3	<0.01	83.0	0.1	4.7	1667	-
	(b)		0.6	1.6	0.9	0.9	0.9	21.7	27.8	0.5
B			1	<0.01	<0.01	0.01	0.8	33.8	26.9	-
				<0.01	1.0	0.5	0.1	605	46.3	176.6
C				1	0.6	0.9	0.3	1.2	1.0	-
					4.0	1.8	0.1	10.8	1.9	1.0
D					1	0.7	0.9	0.7	0.9	-
						15.0	0.01	421.6	43.8	41.7
E						1	1.4	42.9	7778	-
							0.2	275	42.5	13.3
F							1	1.05	1.4	-
								0.1	0.2	0.1
G								1	46.6	-
									5.0	0.8
I									1	-
										5.0
J										1

e.g. tsA x tsG gave values of 5.0, 5.9, 10.2 and 21.7 and for tsB x tsE the values were consistently low at 0.5, 0.7, 0.8, 1.6 and 1.6. The variability could be due to slight fluctuations of temperature which invariably occur in the opening and closing of incubator doors; to the physiological state of the cells at any one time or to variation in the input multiplicities in terms of p.f.u.:particle ratios. Until this variability is fully understood and can be controlled, it results in the need to consider the combined data from a series of experiments. For this reason we present in Table 12 the geometric mean of the complementation indices at 36° and 38° from several experiments. At 38° the indices ranged from about 1.0 (tsA x tsJ, tsB x tsE, tsG x tsJ) to greater than 100 (tsB x tsI and tsB x tsJ). Considering the parallel data at 36° it can be seen that the indices were in general lower. They range from about 1.0 to over 50. Bearing in mind the leakiness of mutants tsC and tsF the C.I. values at 38° are all increased at least 2-fold above the control values with the exception of tsA x tsJ, tsB x tsE and tsG x tsJ. Using the 2-fold criterion for these three exceptions we observed that at 36° the cross tsA x tsJ showed a high complementation index, although at this temperature several of the other crosses no longer gave evidence of significant complementation. This is no doubt largely because several mutants are leaky at 36°. Thus only tsB x tsE and tsG x tsJ gave no evidence of complementation at either

Table 12. Geometric Mean of the Complementation Indices from Several Infectious Centre Assays.

ts Mutant		A	B	C	D	E	F	G	I	J
A	(a)	1	1.4	3.5	7.7	16.6	2.0	3.7	65.5	11.1
	(b)	1	2.1	2.0	9.8	8.5	3.6	9.0	36.4	0.7
B			1	2.1	1.0	1.1	1.3	2.2	7.3	2.1
			1	2.6	6.7	0.7	19.0	34.7	161.9	161.2
C				1	2.9	1.5	0.9	1.4	3.2	2.9
				1	3.8	2.0	12.9	4.7	5.8	3.3
D					1	3.1	2.4	1.0	1.4	0.8
					1	30.9	4.5	27.1	11.0	7.0
E						1	1.3	6.4	10.6	66.1
						1	3.1	38.1	51.9	55.6
F							1	0.6	2.2	3.9
							1	3.2	2.1	3.3
G								1	2.1	1.0
								1	2.3	1.6
I									1	8.6
									1	18.7
J										1
										1

(a) 36° mean

(b) 38° mean

temperature. (The efficiency of plating of infectious centres was on average about 30% and there was only a marginal difference in the efficiency of plating of infectious centres of the wild type at 31°, 36° and 38°).

C. Progeny yield test.

As a second approach to the study of complementation groups we compared the burst sizes from cell populations multiply infected with pairs of ts mutants when grown at the non-permissive temperature (38°) with control single parent infections.

This type of experiment gives a more accurate assessment of complementation of function, since it is not possible to exclude the involvement of recombination as a contributing factor to the production of an infectious centre plaque. Also leakiness is of less significance in a yield experiment. The results of one such experiment in which all the infections were carried out at the same time is given in Table 13. The results show that the majority of pairwise combinations gave positive complementation at 38° (taking values of 5 or greater as positive). The complementation indices ranged from 3-900. Five of the pairs tsA x tsC, tsA x tsE, tsA x tsF, tsB x tsE and tsG x tsJ showed low complementation indices at 38°C.

Again in this type of experiment as well as in the infectious centre assay the level of complementation was often variable from one set of experiments to another. For this reason it was considered

Table 13.

Yield Experiment (1)

4×10^6 BHK21/C13 cells were infected at a m.o.i. of 5 p.f.u. of each of two ts mutants. After adsorption in suspension the infected cells were pelleted, the supernatant discarded and the cells resuspended in 4 ml of E. T. C. The cells were then incubated in plastic dishes at 38°C for 20 hours. After incubation the cells plus medium were harvested and the progeny virus assayed by the standard plaque assay at 31° and 38°C. The complementation index was calculated as

$$CI = \frac{(A+B)^{31°} - (A+B)^{38°}}{\frac{1}{2}(A^{31°} + B^{31°})}$$

where $(A+B)^{31°}$ is the average titre of the progeny virus from two plates incubated at 31°C.

Table 13. Yield Experiment (1)

Complementation Indices

	A	B	C	D	E	F	G	I	J
A	1	10	4	106	3	3	5	30	10
B		1	16	10	4	26	80	20	900
C			1	50	12	56	42	50	75
D				1	26	6	100	50	11
E					1	170	27	6	30
F						1	130	22	44
G							1	59	3
I								1	20
J									1

necessary to consider the combined data from a series of experiments. The data presented in Table 14 show the geometric mean complementation indices from a series of experiments. The results show that by analysing the data in this way several combinations gave indices less than 5 viz. tsA x tsC, tsA x tsE, tsA x tsF, tsA x tsG, tsA x tsJ, tsB x tsC, tsB x tsD, tsB x tsE, tsG x tsI and tsG x tsJ.

It was possible that recombination was playing a considerable part in the yield experiments at the non-permissive temperature. To determine if this was the case we measured the ts+ progeny from mixed infections at the non-permissive temperature and this was taken into account in the calculation of complementation indices (Table 15). It became apparent from the results that in most cases recombination was playing a very small part in the complementation experiments. If recombination as opposed to complementation had been the major cause of the increased titres in mixed infections then the yields would have been very low as the majority of the cells at the non-permissive temperature would not have produced any progeny.

D. Plaque morphology test.

In the case of the cross tsG x tsJ no increase in either numbers of infectious centres or burst size was observed in most cases at 36° or 38° compared to the controls. Using our third approach it was found that in an infectious centre assay when the cross contained the syn, syn+ alleles in addition to the ts markers i.e. tsG syn x tsJ syn+

Table 14. Geometric Means of the Complementation Indices Calculated From Several Progeny Yield Tests

ts Mutant	A	B	C	D	E	F	G	I	J
A	1	4.7	3.2	22.8	1.1	2.0	2.7	14.5	3.7
B		1	2.5	2.8	1.1	19.8	14.6	18.7	85.2
C			1	7.0	3.7	10.4	4.9	9.3	5.0
D				1	34.5	9.5	13.9	9.6	17.2
E					1	50.5	42.1	36.1	11.9
F						1	10.1	11.5	14.1
G							1	3.9	2.9
I								1	14.1
J									1

Table 15. Measurement of the Frequency of Recombination in the Yields of ts x ts Crosses When Grown at the npt 38°.

Cross	Titre 38°C	Titre 31°C	% Recombination
tsAtsB	$< 4 \times 10^2$	1.6×10^4	-
tsAtsC	$< 4 \times 10^2$	3×10^5	-
tsAtsD	$< 4 \times 10^2$	1.2×10^4	-
tsAtsF	3.4×10^4	4.8×10^4 (leakiness 2.4×10^4)	20
tsBtsC	$< 4 \times 10^2$	4×10^4	1
tsBtsD	6.3×10^3	6×10^4	10
tsBtsI	$< 4 \times 10^2$	2.8×10^4	-
tsBtsJ	$< 4 \times 10^2$	6.3×10^4	-
tsCtsE	2×10^3	6.3×10^4	3
tsCtsG	4×10^3	2×10^5	2
tsCtsI	4.8×10^3	1.6×10^5	3
tsCtsJ	$< 4 \times 10^2$	4×10^4	1
tsDtsG	$< 4 \times 10^2$	4.8×10^4	-
tsDtsI	1.2×10^3	3.2×10^4	4
tsDtsJ	$< 4 \times 10^2$	4×10^4	-
tsEtsG	10^4	2.4×10^5	9
tsEtsI	2×10^3	8×10^4	2.5
tsEtsJ	$< 4 \times 10^2$	1.6×10^4	2.5

and the reciprocal, the majority of the infectious centres were of a mixed morphology, i.e. partially syncytial and partially non-syncytial. This result would indicate that both genomes were functioning within the infected cell giving rise to each infectious centre at 38°.

As already mentioned complete dominance for syn/syn+ was not observed in the progeny from infectious centre assays of the type ts+syn x ts+syn+.

E. Discussion.

Taking for arbitrary reasons 2 or greater than 2 as evidence of positive complementation, the geometric means of the infectious centre tests show that the majority of pairwise combinations of the temperature sensitive mutants complement at either 36° or 38° or both. Those which failed to show complementation at either temperature by this test were the pairs tsB x tsE and tsG x tsJ. In addition to these two pairs tsA x tsJ failed to show complementation at 38° but did show a positive value of 11.1 at 36°. If we make the assumption that values of 5 or greater and 2, or greater provide suggestive evidence of complementation, the results of the progeny yield test are in very fair qualitative agreement with the infectious centre data.

When considering the mean indices obtained at 38° for both the infectious centre data and the yield experiments, as shown in Table 16 only three crosses consistently fail to show positive complementation

i.e. tsA x tsJ, tsB x tsE and tsG x tsJ. When this data is plotted as illustrated in Figure 6 only one cross tsB x tsE falls outside the limits of both the infectious centre data and the yield data. The cross tsA x tsJ was unusual in not showing complementation at 38° but showing clear evidence of complementation at 36° in infectious centre assays (Table 12). Unfortunately it was not possible to carry out yield experiments at 36° because of the high background growth of single parent infections. However we consider that the mutations tsA and tsJ represent mutations in different cistrons. It is considered that the mixed morphology of the infectious centres obtained in the cross tsG x tsJ is evidence of positive complementation. Therefore the only cross for which no alternative explanation can be found to account for the low complementation indices is the cross tsE x tsB. It is concluded therefore that these two mutations represent alterations within the same gene.

Considering the part played by recombination in the yield experiments it can be seen from the results (Table 15), that in certain crosses no detectable recombination occurred and in others there was a very small amount. The average part played by recombination was 3.0% of the total yield.

On the basis of the results obtained from the complementation analysis the nine temperature sensitive mutants have been assigned to eight complementation groups - tsA, (tsB and tsE), tsC, tsD, tsF, tsG, tsI and tsJ.

Table 16. Comparsion of the Geometric Means From Several Sets of Infectious Centre and Yield Experiments

ts Mutant		A	B	C	D	E	F	G	I	J
A	(a)	1	2.1	2.0	9.8	8.5	3.6	9.0	36.4	0.7
	(b)	1	4.7	3.2	22.8	1.1	2.0	2.7	14.5	3.7
B			1	2.6	6.7	0.7	19.0	34.7	161.9	161.2
			1	2.5	2.8	1.1	19.8	14.6	18.7	85.2
C				1	3.8	2.0	12.9	4.7	5.8	3.3
				1	7.0	3.7	10.4	4.9	9.3	5.0
D					1	30.9	4.5	27.1	11.0	7.0
					1	34.5	9.5	13.9	9.6	17.2
E						1	3.1	38.1	51.9	55.6
						1	50.5	4.1	36.1	11.9
F							1	3.2	2.1	3.3
							1	10.1	11.5	14.1
G								1	2.3	1.6
								1	3.9	2.9
I									1	18.7
									1	14.1
J										1
										1

(a) Infectious Centre Assays Geometric Mean.

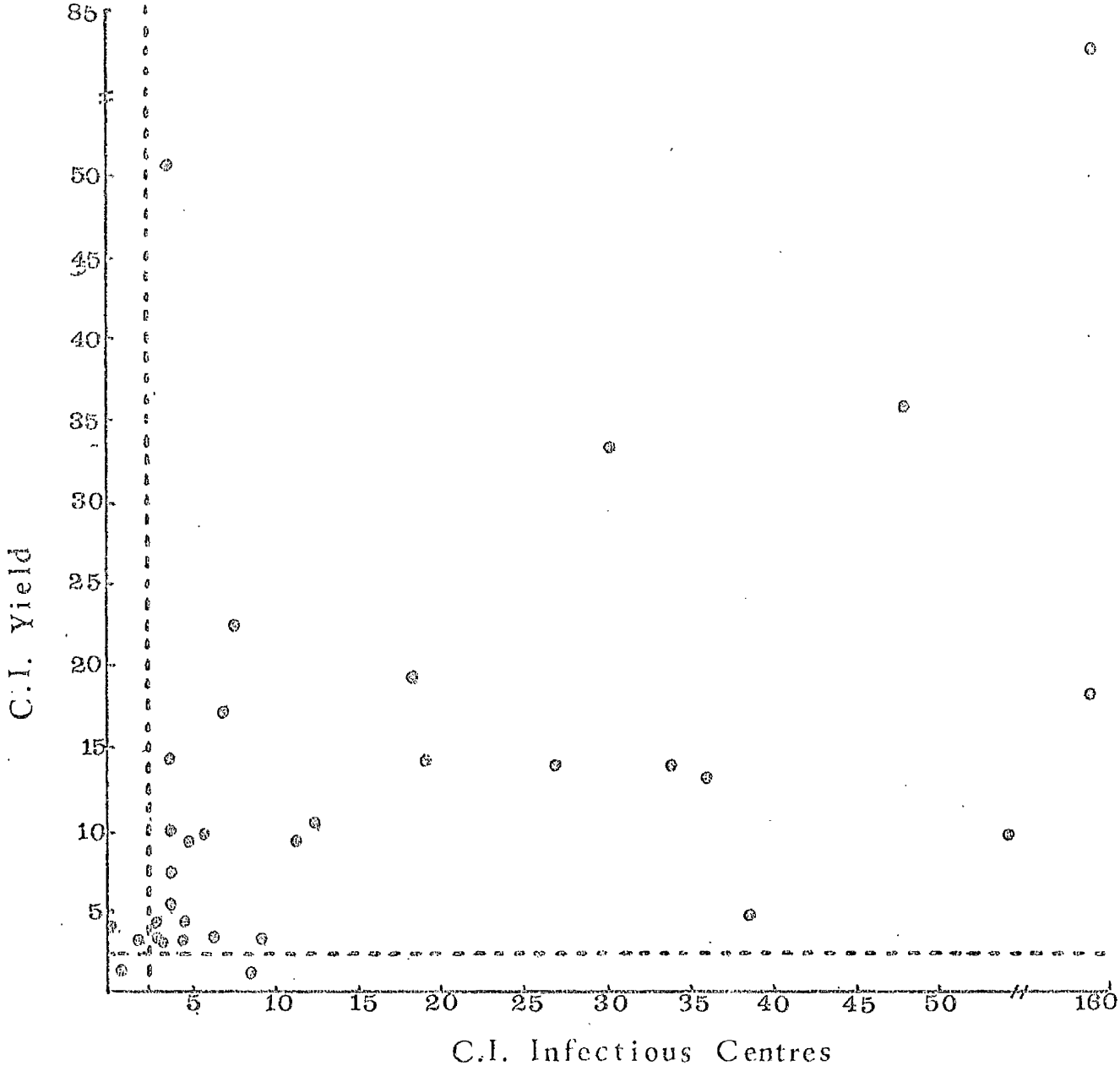
(b) Progeny yield test geometric mean

Figure 6.

Comparison of the Geometric Means From Several Sets of Infectious Centre and Yield Experiments

Plot of results illustrated in Table 16 i.e. mean indices obtained at 38° for both the infectious centre data and the yield experiments.

Figure 6


C.I. Yield
85
50
45
40
35
30
25
20
15
10
5
5
10
15
20
25
30
35
40
45
50
160
C.I. Infectious Centres

III. Genetic Analysis by Recombination.

A. Introduction.

In view of the report of recombination in HSV by Wildy (1955), the availability of ts and syn mutants provided a good opportunity to investigate recombination in HSV. The analysis was approached in two ways.

(1) Two factor crosses of the type tsA x tsB. This was done in order to ascertain if recombination was taking place e. g. the production of ts+ recombinants from two ts parents. If recombination was taking place it would be expected that at the permissive temperature both the parental types and the two recombinant types would grow but at the non-permissive temperature only the ts+ recombinants would grow (Table 17).

(2) Three factor crosses of the type tsAsyn x tsBsyn+ and the reciprocal tsAsyn+ x tsBsyn. This type of three factor cross has the same advantage over two factor crosses in that the output of syn- syn+ in the 31° progeny virus can be monitored. Outputs can be monitored in 2, 3 and 4 factor crosses providing one allele can be easily distinguished from the other. In our system it is the inclusion of the plaque morphology marker which makes this easy. In addition three factor crosses have further advantages:- (a) allows markers to be orientated with respect to their proximity to the plaque morphology marker and (b) can

Table 17. The Growth Properties of the Progeny from a Cross Between Two ts Parents in which Recombination Has Occurred.

		Growth at 31°	Growth at 38°
Parent 1	A ——— +	+	-
Parent 2	+ ——— B	+	-
Recombinant 1	A ——— B	+	-
Recombinant 2	+ ——— +	+	+

calculate recombination frequencies from the same experiment.

B. Two-factor crosses.

The initial experiments undertaken were two-factor crosses of the type tsA x tsB. Table 18 shows the results of one set of two factor crosses which were all carried out at the same time under identical conditions. The results indicate that most of the crosses gave plaques at a higher frequency than the control crosses at 38°. It appeared therefore that the progeny virus from a mixed infection at 31° contained virus which was able to grow at the non-permissive temperature and could therefore for the present be classified as wild type. Self cross controls produced on the whole very little virus which was able to grow at the non-permissive temperature.

The two-factor cross experiments demonstrated that from a mixed infection of two ts parents at the permissive temperature a proportion of the progeny virus was able to grow at the non-permissive temperature. To determine if this progeny virus was truly ts+ or only phenotypically so progeny tests were carried out. To do this mixed infections were carried out in the usual manner at 31° and the progeny titrated at 31° and 38°C. Plaques were then picked from the 38° plates and regrown at 31° and 38°C. Table 19 shows the results of one experiment of this type. The results indicate that in a self cross control of ts+ syn (wild type) the progeny plaques picked from plates grown at 38° contained approximately equal

Table 18

Two-factor cross experiments were carried out for each of the mutants as described (Methods Section 10a). The results show the titre of the total progeny virus (31°) and the ts+ recombinants (38°) in p.f.u. /ml and the recombination frequencies calculated from these results by the formula.

$$RF\% = 100 \times 2\left[(A+B)38°/31° - \tfrac{1}{2}(A38°/31° + B38°/31°)\right]$$

Table 18. Two-Factor Cross Experiment.

Cross	Titre 31° p.f.u. /ml	Titre 38° p.f.u. /ml	RF%
tsA x tsA	1.9×10^6	10^2	0
x tsB	9.5×10^5	2×10^2	0.04
x tsC	3.5×10^5	10^2	0.5
x tsD	1.9×10^6	2.2×10^4	0.18
x tsE	6.3×10^5	$<10^2$	<0.03
x tsF	1.4×10^6	1.4×10^4	2.5
x tsG	1.4×10^6	10^5	1.4
x tsI	3.7×10^6	7×10^2	0.04
x tsJ	1.4×10^6	$<10^2$	<0.01
tsB x tsB	10^6	$<10^2$	0
x tsC	1.2×10^6	1.5×10^4	2.1
x tsD	2.9×10^6	4×10^4	0.27
x tsE	1.1×10^6	$<10^2$	<0.01
x tsF	8.7×10^6	9.5×10^5	1.2
x tsG	1.2×10^6	5×10^4	6.1
x tsI	10^6	4.6×10^4	0.09
x tsJ	1.3×10^6	9.7×10^3	1.6
tsC x tsC	5×10^6	3.5×10^3	0.035
x tsD	1.2×10^6	2×10^5	3.3
x tsE	3.1×10^6	3.2×10^4	2.0
x tsF	2.7×10^6	4.3×10^5	29.4
x tsG	1.8×10^6	2.1×10^5	23
x tsI	2.9×10^6	9.5×10^4	6.1
x tsJ	9.2×10^5	7.1×10^3	1.0
tsD x tsD	2.2×10^6	$<10^2$	0
x tsE	10^6	3×10^4	6.0
x tsF	1.2×10^6	4×10^5	6.6
x tsG	2.1×10^6	1.2×10^4	1.0
x tsI	4×10^6	5×10^4	2.5
x tsJ	3.4×10^5	8×10^2	0.47
tsE x tsE	1.2×10^6	$<10^2$	0
x tsF	1.6×10^6	1.3×10^5	16.1
x tsG	1.1×10^6	2.7×10^4	5.0
x tsI	4.1×10^4	2.5×10^4	1.2
x tsJ	2.5×10^5	1.6×10^3	1.29
tsF x tsF	1.9×10^6	6×10^4	3.0
x tsG	3.3×10^6	1.3×10^6	49.6
x tsI	2.0×10^6	3.6×10^5	34.8
x tsJ	3.7×10^6	3.1×10^5	16
tsG x tsG	4.2×10^6	2.6×10^3	0.06
x tsI	1.9×10^6	8.3×10^4	6.2
x tsJ	6.4×10^6	8×10^3	2.5
tsI x tsI	3.2×10^6	$<10^2$	0
x tsJ	2.7×10^5	9×10^2	0.7
tsJ x tsJ	9.2×10^4	$<10^2$	0

Table 19

Progeny tests.

Plaques picked from plates containing ts+ progeny (38°) from tsX x tsY crosses, were regrown at 31° and 38° to determine if the progeny were truly ts+ or only phenotypically so.

Table 19

Progeny Tests

Plaques from 38°	No. of plaques 38°	No. of plaques 31°	Plaques from 38°	No. of plaques 38°	No. of plaques 31°
tsJ x tsB			ts^{+syn} x ts^{+syn} 17+ x 17+		
1	70	50	1	17	20
2	44	12	2	175	120
3	92	76	3	32	30
4	4	2	4	31	37
5	86	30	5	27	33
6	66	42	6	60	101
7	5	8	7	30	34
8	56	48	8	6	14
9	27	9	9	35	40
10	4	3	10	11	25
tsI x tsG			tsCsyn+ x tsIsyn		
1	4	5	1	2	14
2	10	13	2	23	106
3	1	-	3	0	0
4	19	22	4	3	12
5	8	15	5	71	115
6	11	10	6	12	37
7	3	4	7	3	0
8	-	5	8	4	14
9	30	36	9	3	0
10	44	36	10	15	0
tsF x tsE			tsDsyn x tsEsyn+		
1	5	9	1	30	40
2	213	164	2	54	32
3	18	31	3	7	4
4	600	640	4	17	12
5	7	16	5	2	0
6	56	39	6	20	21
7	3	14	7	40	60
8	15	24	8	25	30
9	31	78	9	1	0
10	172	265	10	0	2

numbers of plaques at the permissive and non-permissive temperatures. It would be expected that if progeny virus from a ts x ts cross which formed plaques at 38° was wild type i.e. ts+, the plaques picked from 38° should contain virus which grows equally well at both temperatures. It can be seen from the results in the great majority of cases this held true. From seventy plaques isolated from 38° plates from six sets of 31° mixed infections, only 10% yielded particles which gave ratios of greater than 1:2, 38°/31° when replated at both temperatures. It would appear therefore that in this system true recombination is taking place, producing stable genotypes with recombinant markers.

As in the complementation analysis there was considerable variation from one set of recombination experiments to another in the actual estimates of recombination frequencies even although conditions were standardised as much as possible. Recombination values did not remain constant from one experiment to another. It was usually the case that if the R. F. value for one particular cross was higher or lower than in a previous experiment then all the crosses gave R. F. values which were higher or lower. In other words there was an overall variation which was not confined to certain particular crosses or crosses involving certain mutants e. g. the R. F. values in two experiments obtained for the crosses tsB x tsF were 15. 7 and 27; tsB x tsJ 3. 3 and 9. 96; tsC x tsG 3. 6 and 18. 7. Most of the other mixed infections in the second

experiment gave higher R. F. values than in the first. There were a few however which gave relatively constant values e. g. tsA x tsD gave 18 and 18. 9 and tsB x tsD 11 and 11. 9.

As in the complementation analysis the reasons for this variation is not completely clear and for this reason we have combined the data from three sets of two factor crosses and taken the geometric mean of the R. F. values from these experiments. Table 20 shows these mean R. F. values. In as much as it is justified ~~in combining~~ to combine the results from three sets of experiments it can be seen that the recombination frequencies ranged from less than 0. 01% for the cross tsB x tsE to about 50% for the crosses tsD x tsF and tsG x tsF.

The two-factor cross data provided certain fundamental information - (1) Recombination was taking place in mixed infections of two ts mutants at the permissive temperature.

(2) The recombination frequencies were measurable and ranged from about 0. 01% to 50%.

(3) The range in R. F. values for different pairs of ts mutants indicated linkage between the nine ts mutants. Certain conclusions could be drawn about the relationship of the ts markers to one another viz. tsD and tsF and tsG and tsI seemed to be the least closely linked: tsB and tsE seemed to be the most closely linked: tsC was nearest to tsF: tsG was nearest to tsD: and tsI, tsA and tsJ were all fairly closely linked.

Table 20. Recombination Frequencies from Two-Factor Cross Data. Geometric Mean of 3 Sets of Data.

Cross ts x ts		RF(%)(Geometric Mean of 3)
A	A	0
	B	0.08
	C	14.49
	D	9.22
	E	2.0
	F	28.7
	G	7.69
	I	0.136
	J	2.28
B	B	0
	C	2.203
	D	11.86
	E	<0.013
	F	20.94
	G	0.164
	I	0.385
	J	5.19
C	C	0
	D	12.16
	E	3.53
	F	8.17
	G	5.84
	I	13.03
	J	4.66
D	D	0
	E	4.63
	F	49.5
	G	8.2
	I	14.86
	J	13.65
E	E	0
	F	18.03
	G	0.49
	I	5.27
	J	2.88
F	F	0
	G	49.8
	I	11.1
	J	8.58
G	G	0
	I	0.38
	J	3.1
I	I	0
	J	3.1
J	J	0

RF(%) Ascending Order ts x ts		
B	E	<0.013
A	B	0.08
A	I	0.136
B	G	0.164
B	I	0.38
G	I	0.38
E	G	0.49
I	J	1.5
A	E	2.0
B	C	2.2
A	J	2.28
E	J	2.88
G	J	3.1
C	E	3.53
D	E	4.63
C	J	4.66
B	J	5.19
E	I	5.27
C	G	5.84
A	G	7.69
C	F	8.17
D	G	8.2
F	J	8.38
A	D	9.22
F	I	11.1
B	D	11.86
C	D	12.16
C	I	13.03
D	J	13.65
A	C	14.49
D	I	14.86
E	F	18.03
B	F	20.94
A	F	28.7
D	F	49.5
F	G	49.8

C. Time course of production of ts+ recombinants.

Having established that recombination was taking place between pairs of ts mutants to form wild type virus, the time course of production of ts+ recombinants was investigated. This experiment was carried out as for a one-step growth experiment. Cells were infected at a m. o. i. of 5 p. f. u. of each of two mutants. After adsorption and neutralisation of unadsorbed virus the infected cells were incubated at 31°C. Plates were harvested at intervals from 0-30 hrs post infection and the progeny titrated at 31° to determine the total virus produced and at 38° to determine the proportion of ts+ recombinants. Recombination frequencies were calculated using the standard formula. Figure 7 shows the results obtained from three crosses - tsB x tsI, tsD x tsI and tsB x tsD. The results are plotted as % R. F. against time of incubation. It can be seen from the results that for the crosses tsB x tsI and tsD x tsI, ts+ progeny first appeared at 9 hrs post infection and increased to 24 hrs and then began levelling off to 30 hrs post infection. For the cross tsD x tsB, ts+ progeny first appeared at 6 hrs post infection and increased to 30 hrs post infection. The recombination frequencies increased from 0. 2% at 9 hrs to 2. 2% at 30 hrs for the cross tsB x tsI from 0. 32% at 6 hrs to 2. 4% at 30 hrs for the cross tsB x tsD and from 0. 22% at 9 hrs to 4. 8% at 30 hrs for the cross tsD x tsI. (These values would be consistent with the location of the three markers on the linkage map illustrated in Figure 8). For each of the crosses total new infectious virus first appeared at 6 hrs post

Figure 7

Time Course of Production of ts+ Recombinants

The experiments were carried out as for a one-step growth experiment. Cells were infected at a m.o.i. of 5 p.f.u./cell of each of two ts mutants. The infected cells were incubated at 31° and harvested at intervals from 0-30 hrs. post infection and the progeny assayed at 31°, to determine the total progeny and at 38° to determine the proportion of ts+ recombinants.

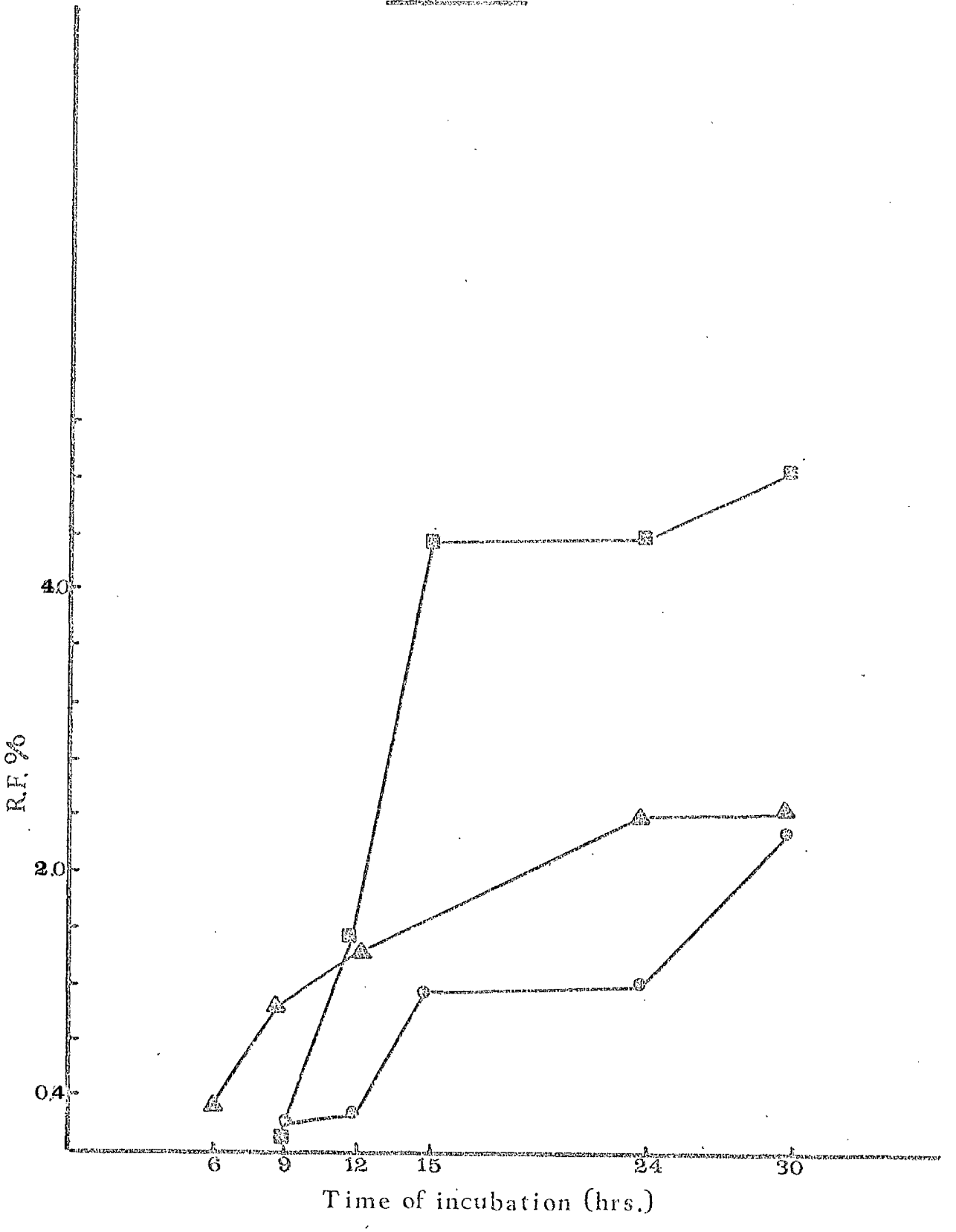
The data are plotted as % R.F. against time of incubation.

tsB x tsI ●——●

tsB x tsD ▲——▲

tsD x tsI ■——■

Figure 7



infection and increased to 24 hrs post infection. It would appear therefore that the production of recombinants follows fairly closely the time course of production of total progeny virus and there would not appear to be any lag and therefore any build up of pools of genomes before the production and release of recombinants. The increase in R. F. values with time may have some bearing on the variation in R. F. values observed from one experiment to another.

D. Three-factor crosses.

Three-factor crosses supply four types of information:-

(1) Output ratios.

Three-factor crosses of the type tsA syn x tsB syn+ and the reciprocal tsA syn+ x tsB syn were carried out for all the possible combinations of mutants. The results of one set of simultaneously performed three-factor crosses are given in Table 21. As mentioned above it is possible to monitor the output ratios of syn:syn+ in the progeny virus at 31° and it was found that the ratio of syn:syn+ or vice versa could be as high as 10:1 in spite of crosses being made with input ratios of 1:1 p.f.u. When the input multiplicities are equal i.e. 1:1 syn:syn+, it would be expected that the total progeny at 31° should also contain equal numbers of syn and syn+, all other factors being equal. In the crosses the input multiplicities are controlled as strictly as possible to 2×10^7 p.f.u. of each mutant into 4×10^6 cells, but it was not always the

Table 21. Recombination by Three-Factor Crosses

tsXsyn x tsYsyn+		31° plaque numbers $n_1/n_2/n_3$*	31° titre x 10^{-6}	38° plaque numbers $n_1/n_2/n_3$*	38° titre x 10^{-3}	RF(%)	ts marker+ closer to sy
A	-	20/0/0	32	0/0/0	<0.1	<0.0001	-
-	A	0/15/0	24	0/0/0	<0.1	<0.0002	-
A	B	240/240/0	20	20/10/2	32	0.32	B
A	B2(E)	10/5/0	23	22/7/1	30	0.26	B2
A	C	210/290/8	20	30/160/10	200	2.0	A
A	D	428/220/32	27	18/34/2	54	0.40	A
A	F	110/220/66	17	15/135/3	1280	15.0	A
A	G	215/200/19	8.5	4/10/0	280	6.4	A
A	I	~~215/200/12~~ 190/210/12	~~17.~~ 16	~~27/4/0~~ 5/30/4	~~31~~ 39	~~0.36~~ 0.45	A
-	B	0/6/0	9.6	0/0/0	<0.1	<0.0005	-
B2(E)	-	16/0/0	25	0/0/0	<0.1	<0.0002	-
-	B2(E)	0/91/0	29	0/0/0	<0.1	<0.0002	-
B2	A	250/200/20	23	5/30/0	35	0.3	B2
B2	B	80/32/2	4.5	0/0/0	0.1	0.005	
B2	C	169/222/9	4.0	11/47/2	0.6	3.0	B2
B2	D	57/30/3	2.8	8/47/3	48	3.1	B2
B2	F	80/230/10	12	20/80/2	1020	17	B2
B2	G	24/42/3	2.6	6/28/2	36	2.8	B2
B2	I	160/52/3	8.5	10/40/3	53	1.3	B2
C	-	8/0/0	12	200/0/0	20	0.08	-
-	C	0/13/0	20	0/30/0	3	0.0007	-
C	A	300/190/16	16	150/48/5	203	2.5	A
C	B	240/230/12	19	380/28/20	430	4.5	B
C	B2	50/28/2	3.1	280/180/20	480	3.1	B2
C	D	115/120/12	10	23/2/1	260	5.2	D
C	F	110/200/15	8.7	11/51/3	650	15.0	C
C	G	180/200/15	16	190/70/12	272	3.4	G

Table 21 contd.

tsXsyn x tsYsyn+		31° plaque numbers	31° titre x 10^{-6}	38° plaque numbers	38° titre x 10^{-3}	RF(%)	ts marker+ closer to s
C	I	181/150/7	34	231/72/5	310	1.82	I
D	-	2/0/0	3.2	12/0/0	1.2	0.0002	-
-	D	0/16/0	25	0/0/0	<0.1	<0.0002	-
D	B	86/78/4	6.7	95/55/10	150	4.5	B
D	B2	49/22/0	2.2	23/9/0	34	3.2	B2
D	C	80/80/0	6.3	50/90/11	151	4.8	D
D	F	110/115/4	9.1	20/90/9	119	26.0	D
D	G	97/150/3	9.6	98/20/4	122	3.0	G
D	I	80/100/10	7.4	170/30/18	218	5.9	I
F	-	13/0/0	20	100/0/0	10	0.025	-
-	F	0/10/0	16	0/30/0	3	0.001	-
F	A	190/180/15	12	40/16/3	590	9.8	A
F	B	160/152/0	12	50/14/2	660	11.0	B
F	B2	110/98/6	8.5	38/20/4	620	14.6	B2
F	C	90/240/16	14	11/0/0	110	15.7	C
F	D	120/110/7	10	115/25/5	1450	29.0	D
F	G	112/200/12	12	100/20/10	1300	21.6	G
F	I	200/460/15	26	160/40/8	208	16.0	I
G	-	20/0/0	32	0/0/0	<0.1	<0.0001	-
-	G	0/14/0	20	0/0/0	<0.1	<0.0002	-
G	A	210/215/20	17	48/3/2	530	6.3	A
G	B	288/160/16	14	50/14/2	660	9.5	B
G	B2	160/52/3	8.3	60/20/5	85	2.1	B2
G	C	120/171/16	12	140/200/20	360	6.0	G
G	D	12/10/0	35	12/28/5	450	2.6	G
G	F	98/197/6	8.3	20/60/9	890	21.5	G
G	I	33/31/0	60	80/35/5	120	4.0	I
I	-	20/0/0	32	0/0/0	<0.1	<0.0001	-
-	I	0/20/0	32	0/0/0	<0.1	<0.0001	-

Table 21 contd.

tsXsyn x tsYsyn+		31° plaque numbers	31° titre x 10^{-6}	38° plaque numbers	38° titre x 10^{-3}	RF(%)	ts marker+ closer to sy
I	A	215/200/12 ~~190/210/12~~	17 ~~16~~	27/4/0 ~~5/30/4~~	31 ~~39~~	0.36 ~~0.48~~	~~A~~
I	B	190/220/12	11	220/23/10	253	4.6	B
I	B2	80/200/10	11	40/5/5	500	0.91	B2
I	C	115/120/9	9.6	20/60/1	81	1.7	I
I	D	81/93/4	3.7	25/70/5	990	5.4	I
I	F	17/15/1	50	7/28/2	3600	14.4	I
I	G	18/10/2	48	30/90/5	125	5.2	I
J	-	18/0/0	28	0/0/0	<0.1	<0.0002	-
J	A	185/290/25	19	4/21/4	280	3.0	J
J	B	180/130/15	12	30/50/10	900	1.5	J
J	B2	89/72/8	67	10/22/5	37	1.1	J
J	C	180/110/20	12	30/140/10	180	3.0	J
J	D	73/112/5	7.6	20/80/6	106	2.8	J
J	F	104/112/9	8.9	8/58/8	740	16.7	J
J	G	27/15/4	72	10/60/5	750	2.1	J
J	I	18/15/4	59	50/120/11	181	0.61	J

* $n_1/n_2/n_3$ is always given so that n_1 gives the number of syn plaques, n_2 the number of syn+ plaques and n_3 the number of mixed syn, syn+ plaques on the same plate.

\+ According to standard genetic practice, the syn allele predominating in the selected ts+ recombinant class (column 5) is located closer to that ts mutant which entered the cross with the alternative syn allele.

Three-factor crosses were carried out as described (Methods Section 10b). All the possible combinations (with the exception of those involving tsJ syn+) were performed in the same experiment. The progeny was titrated at 31° to determine the total yield and at 38° to determine the ts+ progeny. The recombination frequencies were calculated as illustrated (Methods Section 10a).

case that the progeny virus contained equal numbers of syn and syn+ plaques at 31°. Being unable for the present to fully explain this unequal output but realising that it must be a reflection of the pool ratio of syn:syn+ which could be due to (a) inaccurate initial measurement of titre (b) dilution errors, (3) fall off in the fridge of the titres at different rates and (d) slight differences in the pH of the medium, it was decided that only mixed infections which gave not greater than 3:1, 1:3 syn:syn+ output should be used in the construction of a linkage map. Table 21 is this selected data. In some crosses it was found necessary to alter the input ratios to 1:3 or 3:1 tsA:tsB in order to achieve 1:1 output. This was anticipated from the original experiments. (Output ratios at 31° are given in column 3).

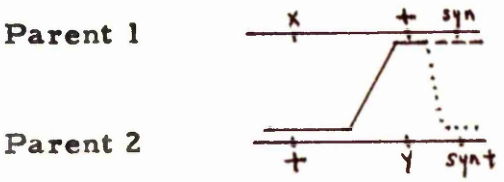
(2) Orientation of markers independently of map distances.

Three-factor crosses allow markers to be orientated independently of map distances i.e. R.F. values. The rationale for the orientation of markers is illustrated in Table 22. Column 8 of Table 21 shows how the plaque morphology marker was located with respect to the two ts markers taking part in the crosses for that set of data e.g. for the cross tsA syn+ x tsF syn which gave a 1:1 ratio at 31°, the ratio of syn:syn+ at 38° was greater than 3:1 and so from this it can be deduced that the plaque morphology marker is located nearer to tsA than tsF (Table 22,

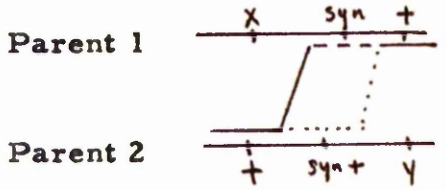
Table 22.

Arrangement 1.

(a)

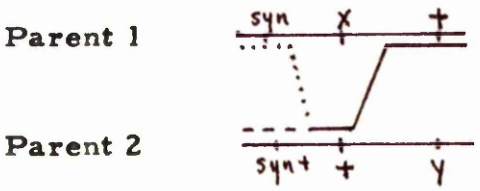


(b)

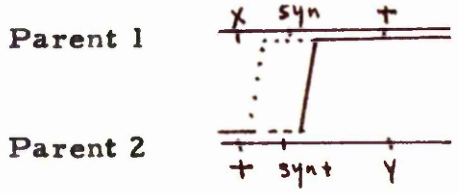


Arrangement 2.

(a)



(b)



——— Selected class

– – – – More frequent

..... Less frequent

In plating the progeny from a three-factor cross experiment at 38°, we are selecting for ts+ recombinants. Making the assumptions that (1) crossovers are random for position, (2) crossovers are equally frequent, (3) crossovers are more frequent when the markers are farther apart than closer together and (4) two crossovers are less frequent than one. When the majority of ts+ recombinants from a cross are syn, then only arrangements 1a and 1b would satisfy the requirements i. e. the plaque morphology marker is closer to tsY than tsX. If the majority of ts+ recombinants are syn+ then arrangement 2a and 2b would satisfy i. e. the plaque morphology marker is located nearer to tsX than tsY. In this way markers can be orientated independently of map distances.

arrangements 1a and 1b).

(3) Recombination frequencies.

The titre of progeny virus at 31° and 38° can be calculated and hence the R. F. values for each of the crosses (Methods Section 10a). Table 21 column 7 shows the R. F. values obtained for that particular set of crosses.

(4) In addition to supplying the above three types of information, it became apparent from the three-factor cross experiments that in addition to the progeny virus forming plaques of the parental type i. e. syn and syn+, there was also a small proportion of plaques of a mixed morphology i. e. partially syncytial and partially non-syncytial. It can be seen from Table 21, column 5 that e. g. the cross tsB syn+ x tsJ syn gave 30 syn, 50 syn+ and 10 mixed in the progeny virus at 38°C.

E. Construction of a linkage map.

As has been stated previously the frequency of ts+ recombinants for a given pair of ts mutants often varied for the same cross, when made at different times. However comparison of the results obtained from the same sets of crosses performed at different times showed that the relative recombination frequencies remained fairly constant. (Compare the present linkage map with the preliminary one referred to by Hay et al., (1971)). Due to this variability, it was thought that

it would be unrealistic to use the recombination frequencies from more than one set of data for the construction of a linkage map, even although the output ratios of syn:syn+ were 1:1. It was decided, therefore, to use the data from one complete set of three-factor crosses which gave not greater than 3:1, 1:3 syn:syn+ at 31°. The ratio 3:1, 1:3 was chosen due to the difficulties in obtaining enough data with a strict 1:1 output from one set of crosses. The reasons for this are not fully understood at present and therefore it was decided to use data with a 3:1 ratio limit. The set of data which was selected for the construction of the linkage map is that presented in Table 21.

Figure 8 is presented as the genetic map of HSV Type 1. The distribution of the markers gives the best fit and the least dubiety based on the results of our experiments. The map was constructed using both the R. F. values and the ratio of syn:syn+ amongst the ts+ progeny.

The plaque morphology marker was located in relation to the ts markers using the ratio syn:syn+ in the ts+ progeny. From Table 21 it can be seen that for the cross tsF x tsD, the plaque morphology marker was located nearer to tsD than tsF, therefore it would be to the right or left of tsD

F syn D syn

For the cross tsF x tsG it is nearer tsG

∴ F syn G syn

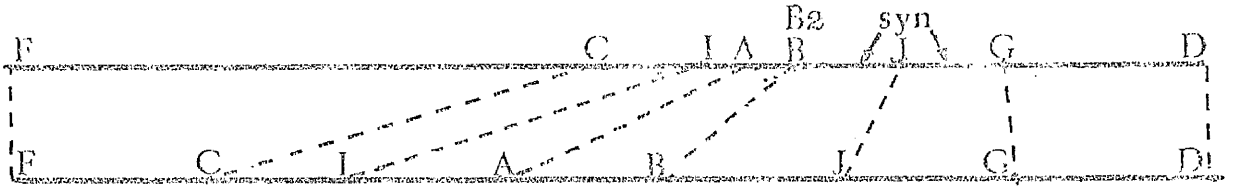
For the cross tsD x tsG it is nearer tsG

∴ F D syn G syn

Figure 8.

Genetic Map of HSV Type 1.

Figure 8



26.1, 29.0

21.5, 21.6

16.7

11.0, 14.6, 17.0

9.8, 15.0

14.4, 16.0

15.0, 15.7

4.8, 5.2

3.4, 6.0

3.0

3.0, 3.1, 4.4

2.0, 2.5

1.7, 1.8

5.4, 5.9

4.0, 5.2

0.61

0.91, 1.3, 4.6

0.36, 0.48

0.4

6.3, 6.6

3.0

0.3, 0.32, 0.26

3.1, 4.1, 4.5

2.1, 2.8, 9.4

1.1, 1.5

2.8

2.1

2.5, 2.6

For the cross tsC x tsI it is nearer tsI

$\therefore$ F C syn I syn I

For the cross tsC x tsJ it is nearer tsJ

$\therefore$ F C I syn J syn

For the cross tsE x tsF it is nearer tsE

$\therefore$ F C I syn J syn E syn

In this way, a map with the order of the markers as follows was built up:

F C D IG A BE J syn

From Table 21, all the locations would confirm unambiguously the position of syn with respect to the ts markers. Table 23 gives the location of syn with respect to the ts markers for all the three-factor crosses which have been carried out. It can be seen that the majority of the orderings were in unambiguous agreement with its location. Using the above map, in combination with the R. F. values between markers, this arrangement was rearranged to give the map presented in Figure 8 e.g. tsD x tsF gave an R. F. value of 29% which was the highest value obtained, therefore tsF and tsD must be the furthest apart markers.

The map spans about 25-30 map units and the distances are reasonably additive. The mutants tsF, tsC, tsD and tsG are the least linked markers; tsB and tsE are the closest linked markers, having never given any recombination at all; tsI, tsA, tsJ, tsB and

Table 23. Location of the Plaque Morphology Marker With Respect for all the Three-Factor Crosses Which Have Been Carried Out.

Cross tsX x tsY		No. of times syn nearer tsX	No. of times syn nearer tsY
A	B	0	5
A	C	5	1
A	D	8	0
A	E	0	4
A	F	5	0
A	G	7	0
A	I	5	2
A	J	(3 not measurable)	1
B	C	5	0
B	D	3	
B	E	(not measurable)	
B	F	4	0
B	G	4	1
B	I	3	2
B	J	0	4
C	D	2	7
C	E	1	5
C	F	5	0
C	G	0	7
C	I	2	4
C	J	0	6
D	E	0	5
D	F	6	0
D	G	2	5
D	I	1	5
D	J	0	4
E	F	4	0
E	G	4	0
E	I	4	1
E	J	1	3
F	G	0	6
F	I	1	4
F	J	1	5
G	I	5	0
G	J	0	4
I	J	0	6

tsE form a cluster spanning about four map units and syn is located either to the right of tsJ, between tsJ and tsG or to the left, between tsJ and tsB. As neither complemention nor recombination has been demonstrated between tsB and tsE, it is thought that these are mutations in the same cistron and therefore tsE is now given the notation tsB2.

F. Discussion.

The following conclusions can be drawn from the recombination experiments:-

(1) Recombination has been shown to occur.

(2) True recombinants have been shown to be produced in the recombination process.

(3) Figure 8 is proposed as a provisional linkage map of the temperature sensitive mutants (tsA - tsJ) of HSV Type 1.

(4) The map spans about 25-30 map units.

(5) The mutants tsF, tsC, tsG and tsD are the least linked, tsB and tsB2 are the closest and tsI, tsA and tsJ along with tsB and tsB2 form a group spanning about four map units. Both the two-factor and three-factor cross data would confirm this arrangement.

(6) Recombination has never been demonstrated between tsB and tsB2 and this is in agreement with the complementation results

in which tsB and tsB2 were never shown to complement with each other. It is thought therefore that the mutants tsB and tsB2 were caused by mutations within the same cistron.

(7) The plaque morphology marker syn has been located to one of two sites either between tsJ and tsG or tsJ and tsB.

(8) Mixed syn:syn+ plaques appear among the progeny of crosses involving these two alleles.

It is interesting to note that the mutants tsF, tsI, tsC, tsG, tsD have all been found to be DNA positive; tsA, tsJ and tsB to be DNA negative and tsB2 seems to synthesise a small amount of DNA (Mechie, et al., 1972).

IV. Genetical and Physical Analysis of Mixed Plaques.

A. Introduction.

As was mentioned in Results Section 3A plaques of a mixed syncytial/non-syncytial character were observed on plates containing progeny virus from crosses in which one parent was syn and the other syn+. It appeared from direct observation of plaque morphology that the plaques were probably not the result of the overlap of syn and syn+ plaques which were developing in close proximity. These mixed plaques were reminiscent of the mottled r/r+ plaques observed by Hershey and Chase (1951), in the progeny of T4 crosses involving the r and r+ plaque morphology markers. These mottled plaques were found to be produced by heterozygous phage particles which are involved in the recombination process in the phage T4. The possibility existed that the mixed plaques of HSV could also function in recombination. For this reason a physical and genetical analysis of mixed plaques was undertaken.

B. Occurrence of mixed plaques.

In crosses in which one parent was syn and the other syn+, regardless of the other markers, e. g. ts syn x ts syn+, ts+ syn x ts syn+ and ts+ syn x ts+ syn+ it became apparent that the plaques formed by the progeny virus were not only syn and syn+ but also syn/syn+ (Photograph 2). It appeared therefore that mixed plaques were a

Photograph 2 mixed plaque

general effect of mixed infections involving syn and syn+. They were also a fairly constant feature of recombination experiments and appeared both amongst the total progeny and the ts+ recombinant progeny. The proportion among the ts+ recombinants seemed to vary depending on the ts markers taking part in the cross and in general the proportion in the ts+ progeny was higher than in the total progeny. Various possibilities could account for the occurrence of mixed plaques:-

(1) Overlap of syn plaques with syn+ plaques.

(2) Clumps of virus particles.

(3) More than one viral capsid within the same envelope.

(4) The formation of heterozygotes.

Experiments were designed to attempt to distinguish between these various possibilities. One set of experiments was concerned with clumping and trying to eliminate clumping of particles and another set was concerned with genetic predictions of segregation from clumps and from heterozygotes.

C. Investigation of the part played by overlap of syn and syn+ plaques in the production of mixed plaques.

To determine what proportion of these mixed plaques was due to overlap of pure syn and pure syn+ plaques, mixtures of tsXsyn and tsXsyn+ etc. in equal proportions were assayed to give a wide range from approximately 10 to 500 plaques/plate. The proportion of mixed

plaques was plotted as a percentage of the total number. For comparison the data from all mixed infections was accumulated and the proportion of mixed plaques on any one plate was plotted as a function of the total number of progeny plaques on that plate. Both sets of data are shown in Figure 9. It can be seen from the plot of the proportion of mixed plaques against the total number that no obvious pattern emerged. There appeared to be a wide scatter ranging from about 50% mixed plaques in a total of 50 plaques to 4% mixed in 500 plaques. With the artificial mixture it can be seen that the proportion of mixed plaques increased with increasing total numbers of plaques but at no time even with about 500 plaques/plate was it higher than 4%. It would appear therefore from the data that overlap plays a very small part in the production of mixed plaques. The proportion of mixed plaques amongst the progeny from a cross was in most cases therefore more than could be accounted for by overlap. It should be pointed out that it is usually quite easy to distinguish a truly mixed plaque from a plaque caused by overlap of syn and syn+ plaques. The orientation of the two sections of the plaque is quite different in each case (Photograph 3).

D. Investigation of the part played by virus clumps in the production of mixed plaques.

To investigate the possibility of mixed plaques being due to clumps of particles three types of experiment have been carried out:- sonication, heat inactivation and U. V. inactivation.

Figure 9.

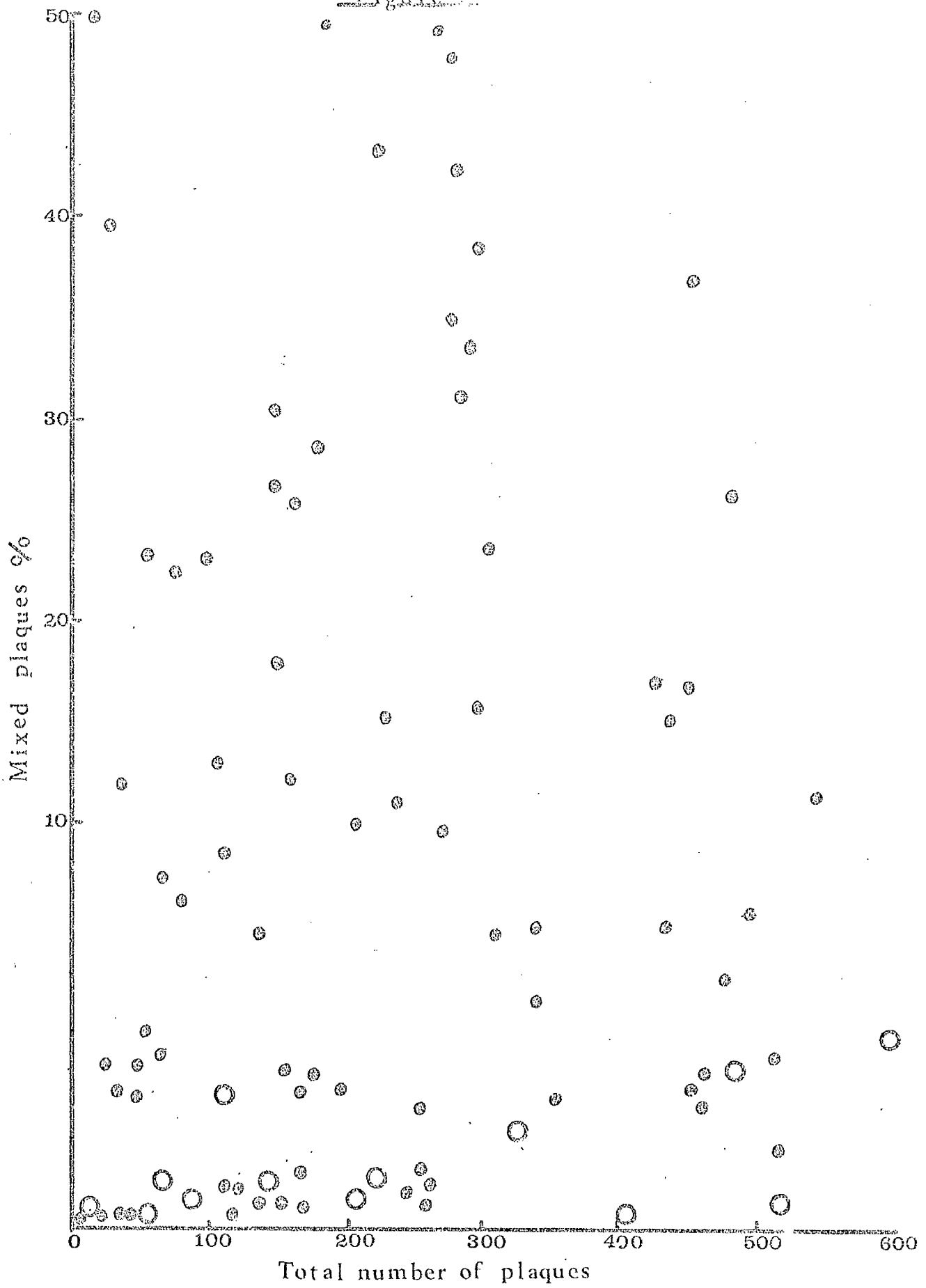
Investigation of the Part Played by Overlap of *syn* and *syn+* Plaques in the Production of Mixed Plaques

Mixtures of *ts*X*syn* and *ts*X*syn+* in equal proportions were assayed to give a wide range of plaques/plate. The proportion of mixed plaques was plotted as a percentage of the total number. The data from all mixed infections was accumulated and the proportion of mixed plaques on any one plate was plotted as a function of the total number of progeny plaques on that plate.

Percentage of mixed plaques on any one plate. ●

Plaques due to overlap of *syn* and *syn+*. O

Figure 8.


50
40
30
20
10
Mixed plaques %
0
100
200
300
400
500
600
Total number of plaques

Photograph 3 overlap

(1) Sonication experiments.

Three-factor crosses of the type tsXsyn x tsYsyn+ were carried out by the standard method. After twenty four hours incubation at 31° the infected cells plus medium were harvested and the progeny virus assayed by the standard method at 31°C. The progeny virus was then sonicated at 60 m amperes for $\frac{1}{2}$, 1, 2, 5, 7 and 10 minutes in a bath sonicator. Samples were withdrawn after each of these times and assayed for total virus and the proportion of mixed plaques at 31°C. Table 24 shows the results of one experiment of this type. The results indicate that except for the slight decrease in the percentage of mixed plaques in the cross tsIsyn x tsGsyn+, there was no significant difference in the percentage of mixed plaques in the total number after sonication treatment for 10 minutes. It would be expected that sonication for this length of time would efficiently break up clumps of particles and hence the percentage of mixed plaques would fall significantly. This result would suggest that the mixed plaques were not due to clumps of particles.

(2) Heat inactivation.

In the heat inactivation experiment a mixed infection was carried out in the usual manner. The progeny virus was titrated and a sample immersed in a water bath at 45°C. Samples were withdrawn at intervals up to 90 minutes and

Table 24. Sonication Experiment

tsIsyn x tsGsyn+	Time of sonication	No. of syn plaques	No. of syn+ plaques	No. of mixed plaques	% Mixed plaques
	0	31	160	22	10
	½	80	400	54	10
	1	62	220	18	6
	2	55	250	21	6
	5	50	226	14	6
	7.5	50	240	16	5
	10	55	300	14	4
tsIsyn+ x tsGsyn					
	0	206	112	22	6
	½	200	120	20	6
	1	205	156	20	5
	2	196	132	22	6
	5	111	82	17	5
	7.5	151	120	16	5
	10	151	102	21	7
tsIsyn x tsDsyn+					
	0	71	34	3	2.8
	½	78	74	4	2.5
	2.5	143	180	12	3.5
	5	120	140	12	4.4
	7	95	114	15	6.7
	10	115	140	15	5.5

assayed for total virus and for mixed plaques. Figure 10a shows the inactivation curves of the total virus and the mixed plaques from a mixed infection of tsIsyn x tsDsyn+. The figure also incorporates the expected theoretical inactivation curve if the mixed plaque was caused by a clump of two particles. This is based on the fact that if a clump of (x) particles of type A and (y) particles of type B is heated sufficiently to reduce the population containing the clump by a factor of 100, the chance that the mixed plaque quality of the clump surviving is at most $(1-0.99^{x})(1-0.99^{y})$ which is very much less than the chance (0.01) that a given virus particle will survive. If the clump is made up of two particles then this chance is $(1-0.99^{1})(1-0.99^{1})$ i.e. 0.0001. If the population is reduced by a factor of 10 the chance of the mixed plaque quality of the clump surviving is (1-0.9)(1-0.9) i.e. 0.01 (Hershey and Chase, 1951). It would appear from the results that the fall off in mixed plaques followed fairly closely the inactivation curve of the total virus population. This was also seen for the mixed plaque population of other mixed infections (Figure 10b). In comparison with the theoretical curve for a two particle clump it is evident that the mixed plaques are not due to two particle clumps and in fact the inactivation curve for the mixed plaques would not fit the theoretical curves for clumps of more than two particles.

Figure 10a.

Heat Inactivation Experiment (Mixed Plaques)

A heat inactivation experiment was carried out at 45°C as described (Methods Section 11). Samples were withdrawn at intervals and assayed by the standard method for total virus and for mixed plaques. The total virus and the mixed plaques are plotted as log surviving fraction against time.

Total virus ●——●

Mixed plaques ▲——▲

Theoretical for 2 particle clump ■——■
(Mixed plaque quality survival)

Figure 10a

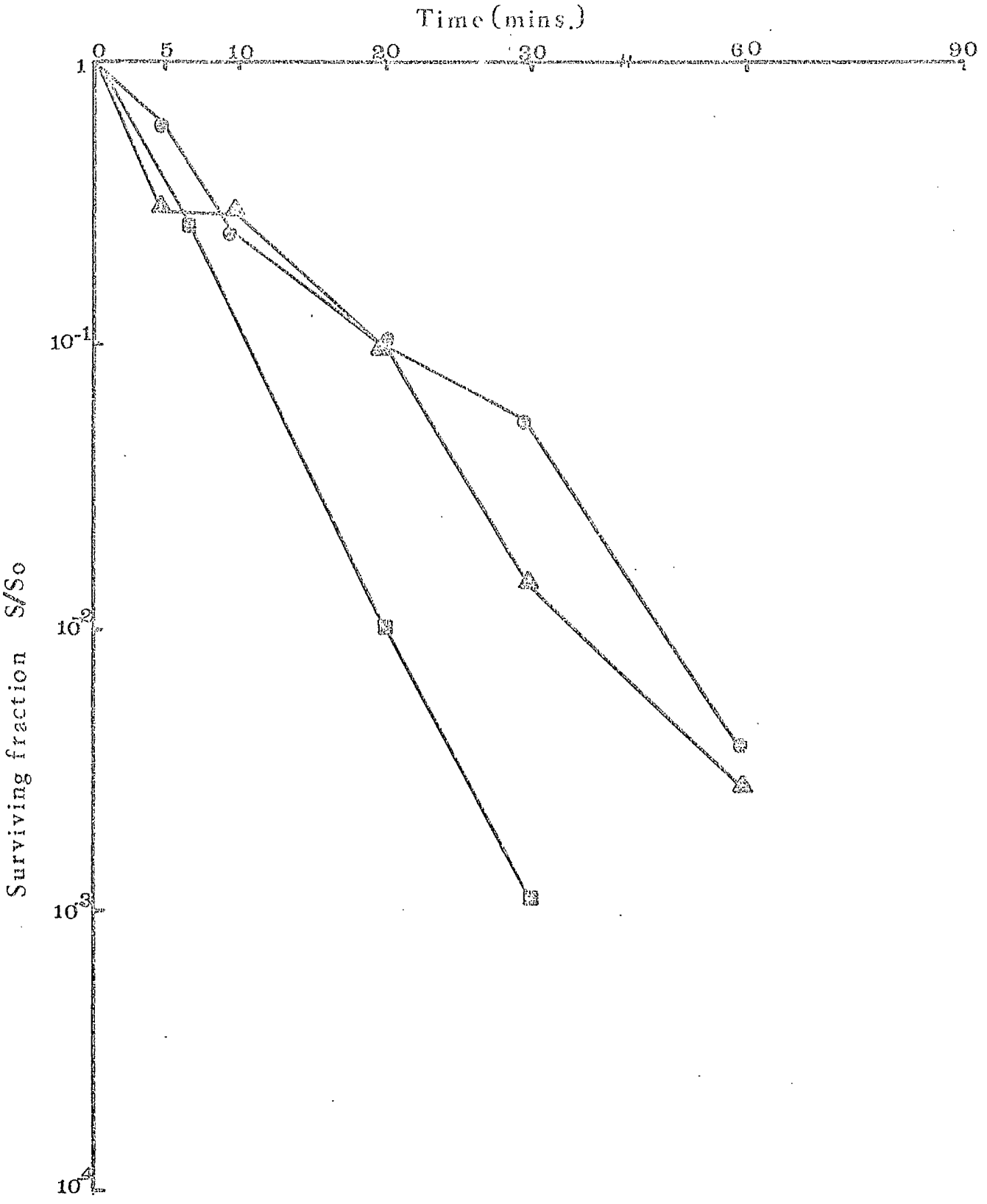
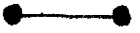

Time (mins.)
0
5
10
20
30
60
90
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Surviving fraction S/So

Figure 10b.

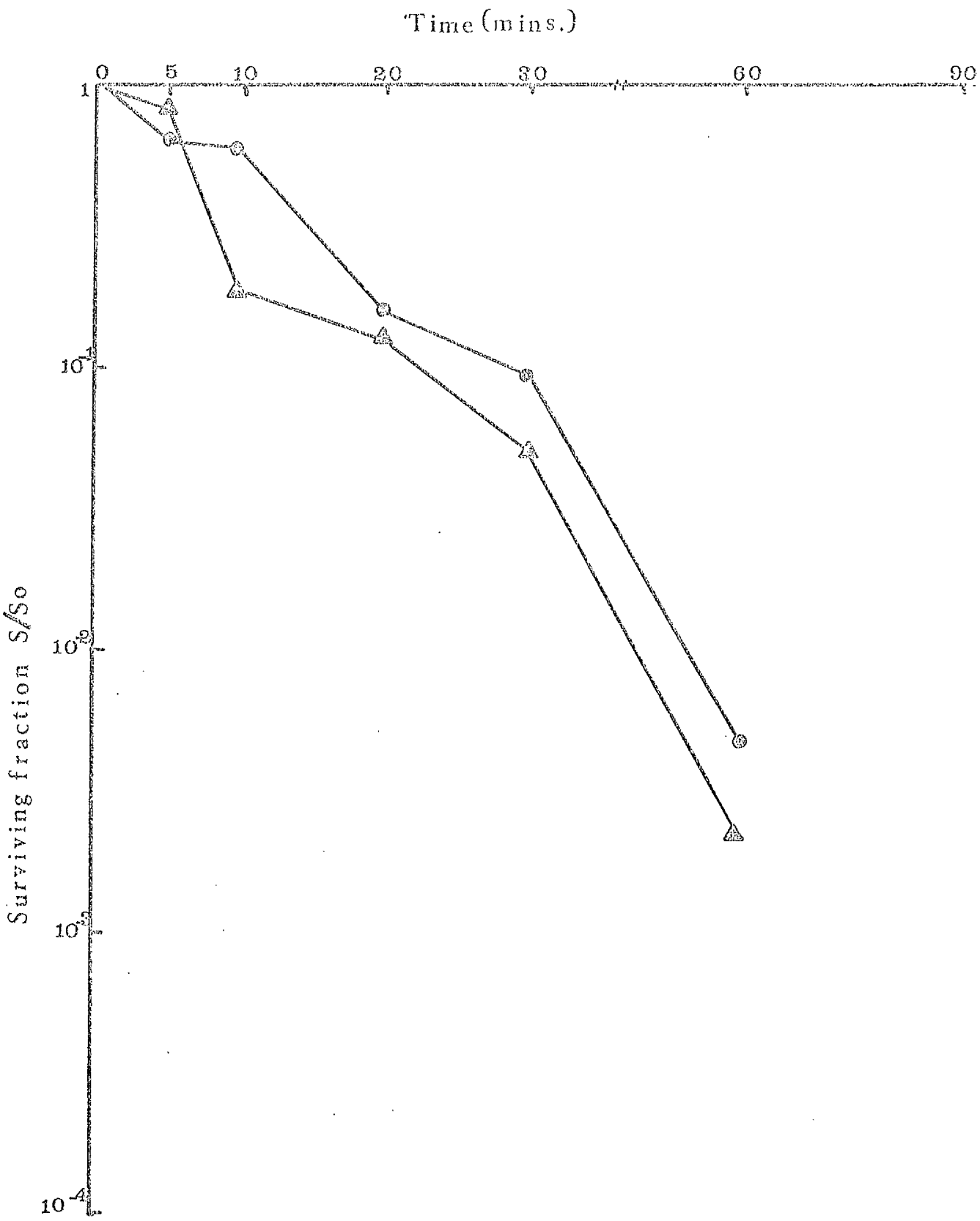
Heat Inactivation Experiments (Mixed Plaques)

Experiments were carried out as for Figure 10a.

Total virus 

Mixed plaques 

Figure 10b


Time (mins.)
0
5
10
20
30
60
90
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Surviving fraction S/So

(3) Ultra-violet light inactivation.

As for the heat inactivation experiments mixed infections of the type tsXsyn x tsYsyn+ were carried out by the standard method. The progeny virus was titrated for total virus and for mixed plaques using the standard assay procedure. The virus was then diluted 1:20 (0.1 ml:1.9 ml) in P.B.S.A. and exposed to a Hanovia U.V. lamp (0.5 amps, 30 watts) for time intervals from 0 to 7 minutes at a distance of 75 cm. (The virus sample was exposed in a 50 cm glass petri dish, the lid being removed during exposure). 0.1 ml samples were withdrawn at intervals and assayed for total virus and for mixed plaques. Figure 11a shows the U.V. inactivation curve obtained from progeny virus from the cross tsDsyn x tsIsyn+. The surviving fraction (Log S/So) both of total virus and mixed plaques is plotted against time of exposure. It can be seen from the results that by 2 minutes there was a 1 log decrease in total virus and by 7 minutes the titre had dropped 2.3 logs. The survival curve of mixed plaques followed fairly closely that of the total virus. By 2.5 mins there was a 1 log decrease and by 7 mins a 1.7 logs decrease. Six U.V. inactivation experiments have been carried out using different mutants and the wild type virus (Figure 11b). The U.V. inactivation patterns obtained were very similar to that shown in detail in Figure 11a. In some cases the curve for mixed plaques was slightly above that of total virus and in other cases it was

Figure 11a.

U. V. Inactivation Experiment

A mixed infection of tsDsyn+ tsIsyn+ was carried out in the standard way. The progeny was diluted 1:20 in P. B. S. A. and exposed to U. V. light for time intervals from 0-7 minutes, at a distance of 75 cm. Samples were withdrawn at intervals and assayed for total virus and for mixed plaques. The surviving fraction (Log S/So) is plotted against time of exposure.

Total virus ●——●

Mixed plaques ■——■

Figure 11a

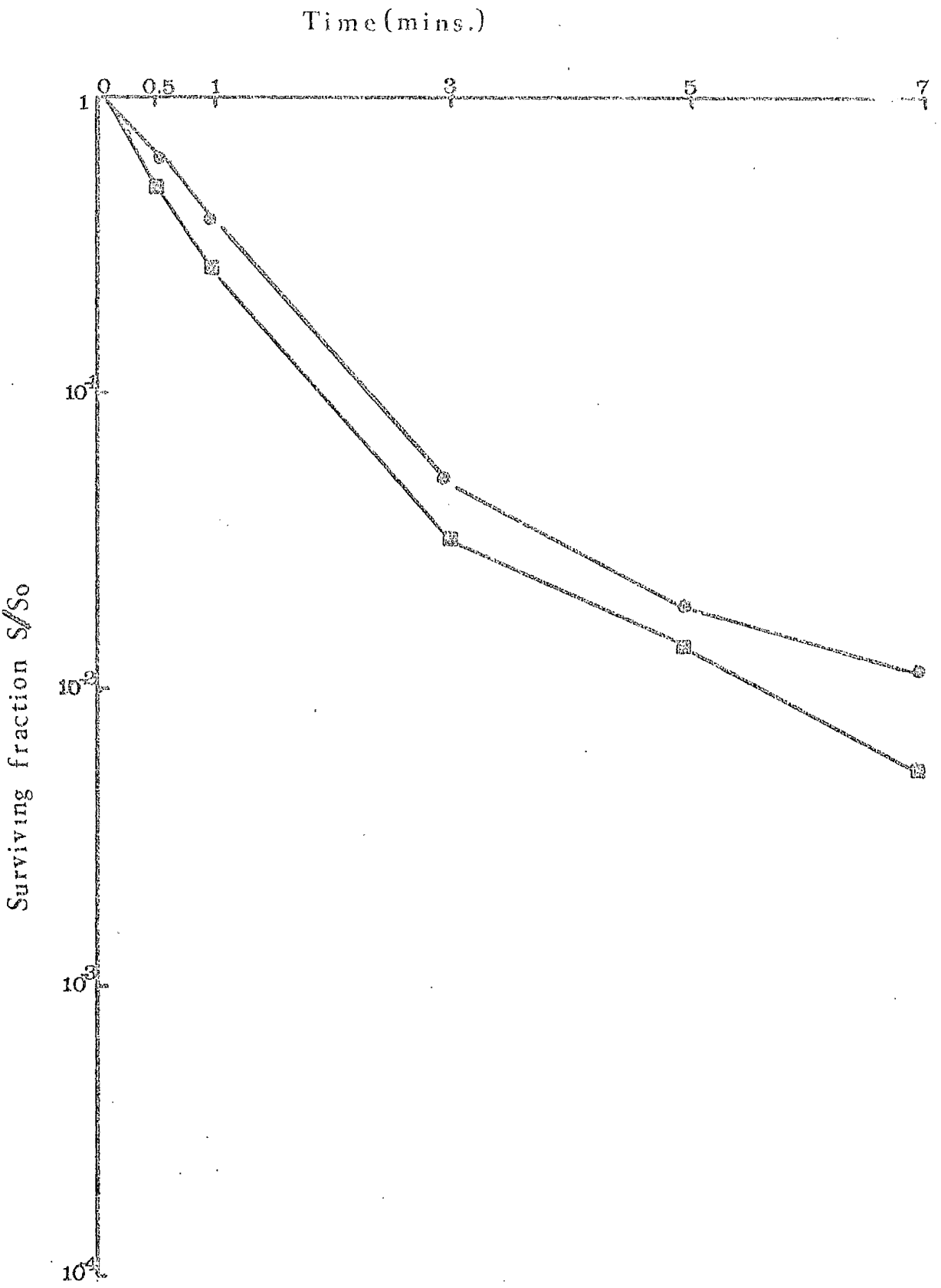


Figure 11b.

U. V. Inactivation Experiment

Experiments were carried out as for Figure 11a.

(1) Asyn x Esyn+

(2) 17 syn x 17 syn+

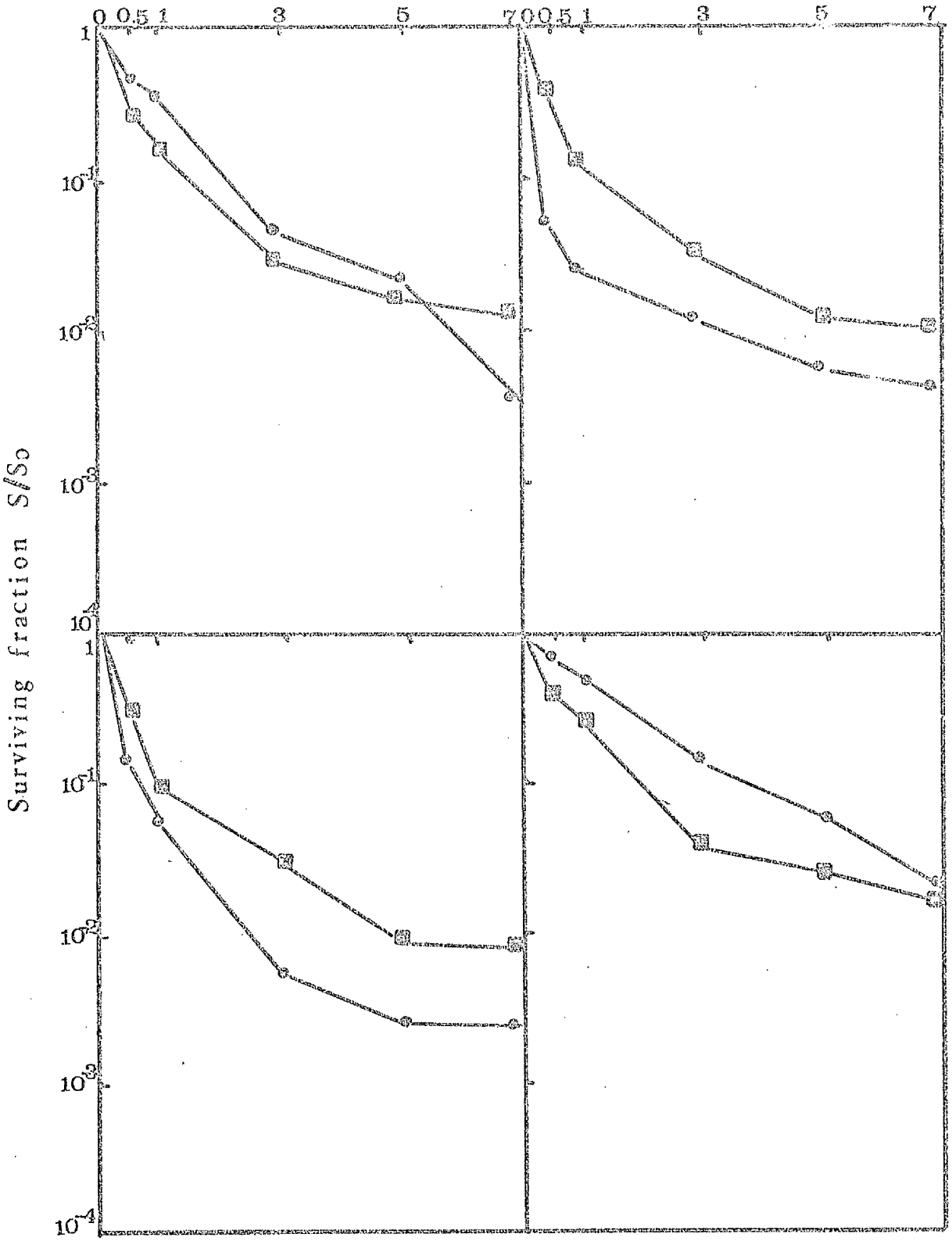
(3) Isyn x Bsyn+

(4) 17 syn x Isyn+

Total Virus ●——●

Mixed plaques ■——■

Figure 11 b


Time (mins.)
0 0.5 1 3 5 7 0 0.5 1 3 5 7
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Surviving fraction S/So

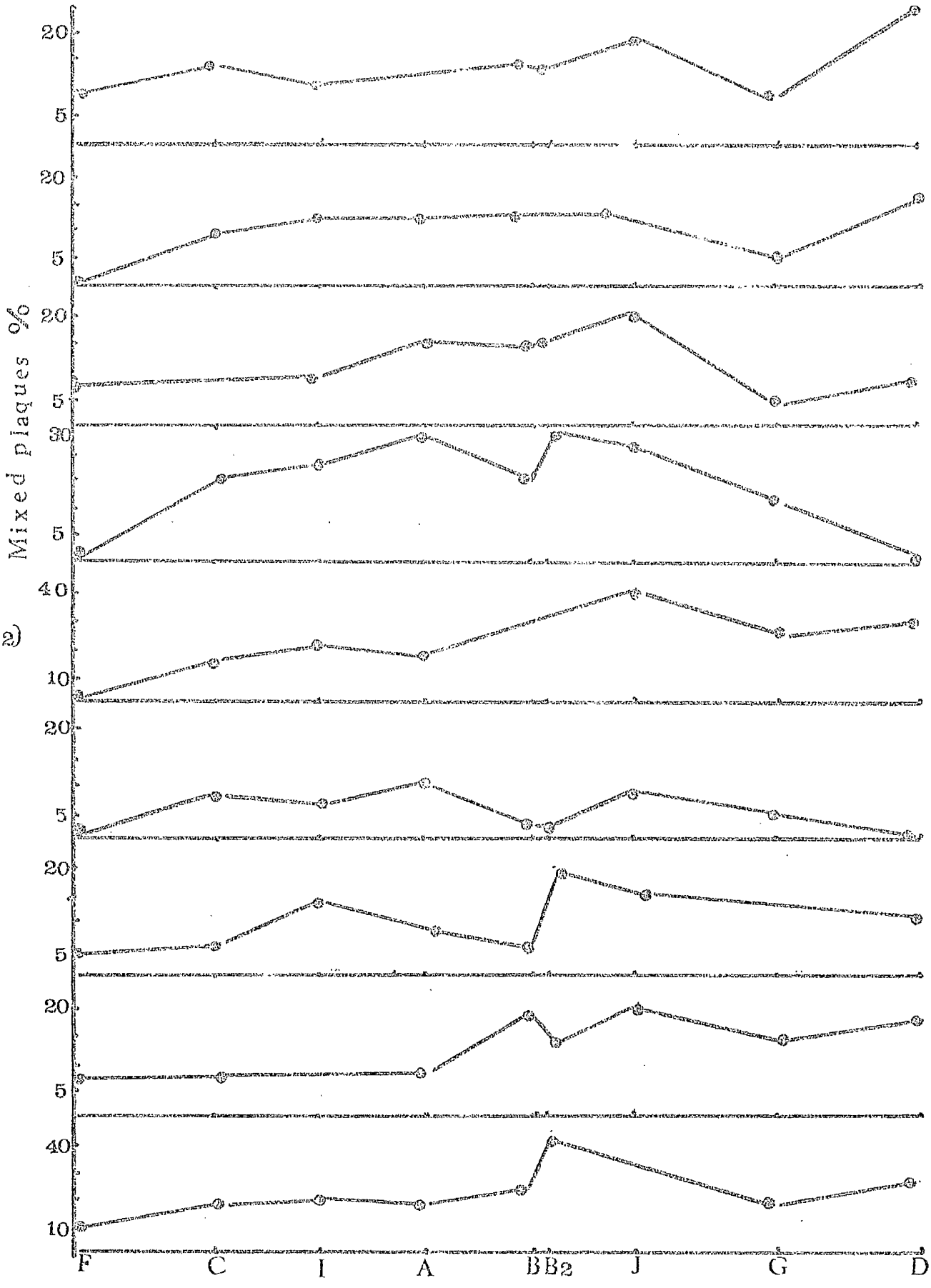
slightly below. It was considered that this slight difference was not significant and was not more than could be accounted for by experimental error. It is concluded therefore that the fall off in mixed plaques does not differ significantly from the fall off in total virus under the conditions of U. V. exposure which were used in the experiment. It would appear therefore that the mixed plaques are not due to clumps of particles and also that they are probably not due to two complete nucleo-capsids within the one envelope, as this would result in a two hit curve. In the experiments which have been carried out, the inactivation curves for mixed plaques at no time showed a shoulder as would be expected for a two hit curve.

It can be seen from Figures 11a and b that as time of exposure increased, the plaque forming ability seemed to become relatively resistant to U. V. light. This was a constant feature of the experiments which have been carried out and it would suggest that multiplicity reactivation might be taking place. This is thought to be the result of infection of the same cell with two inactive particles. The inactive particles are able to co-operate and produce active virus. This phenomenon was first demonstrated by Luria (1947) with T4. If this is happening in our experiments then it would make the test less valid.

E. Frequency of mixed plaques as a function of map location.

Although it appeared from Figure 8 that no pattern was emerging when the percentage of mixed plaques in any one cross was plotted against the total number of plaques it was thought that if the percentage of mixed plaques was plotted as a function of the linkage map that this might provide some further information. To do this all the data for any one cross was accumulated e. g. six tsA x tsC crosses. The values obtained for mixed plaques in the total progeny for each tsA x tsC cross were then averaged and the average value plotted as a function of the map. This was done for all the tsA crosses, all the tsB crosses etc. Figure 12 shows the results of analysing the data in this manner. The results indicate a tendency for the patterns to peak around the region of location of tsJ. For tsA, tsB, tsC, tsD, tsG and tsI the average percentage of mixed plaque values ranged from less than 5 to 20%: for tsB2 and tsJ the values ranged from 10% to 40% and for tsF from less than 5% to 10%. It would appear therefore from this preliminary analysis that there seems to be a relationship between the proportion of mixed plaques produced in a ts syn x ts syn+ cross and the ts mutants i. e. their location relative to syn taking part in the cross. If tsE and tsJ are involved in the cross then a higher proportion of mixed plaques amongst the progeny could be expected than if e. g. tsF or tsG were involved. TsJ and tsB2 are located nearest to the plaque morphology marker and tsF is located furthest away.

Figure 12


Mixed plaques %
20
5
20
5
20
5
30
5
40
10
20
5
20
5
20
5
40
10
F
C
I
A
BB2
J
G
D

F. Segregation of the unselected marker.

It has been established that the large proportion of mixed plaques occurring in the progeny from mixed infections is more than could be accounted for by overlap and it would also seem from the preliminary tests which have been carried out that the mixed plaques were not due to clumps of particles. However it did seem from the accumulated data that the proportion of mixed plaques was related to the mutants taking part in the cross and that the mutants around the location of the plaque morphology marker gave higher proportions of mixed plaques. It seemed a distinct possibility therefore that heterozygosis was involved in the production of mixed plaques. To investigate this further the behaviour of the mixed plaque on regrowing was studied. To do this single non-overlapping mixed plaques from 38° plates were isolated into 1 ml P. B. S. A. and sonicated for approximately one minute. The sonicated virus was then plated on BHK21/C13 cells and incubated at 38° for two days. On fixing and staining the number of plaques was counted and divided into syn, syn+ and mixed categories. The results of one experiment of this type are shown in Table 25. The results indicate that in most cases the mixed plaques when isolated and regrown segregated into the parental syn and syn+ plaques and in some cases they also yielded a small proportion of mixed plaques. In other words there was segregation of the unselected marker. The ratio of one parental plaque type to the other ranged from 1:1 to 10:1 but more than half gave ratios

Table 25. Segregation of Mixed Plaques

Cross	syn	syn+	Mixed
tsG x tsJ			
1	15	0	0
2	9	6	0
3	4	16	0
4	24	0	0
5	8	5	1
6	71	37	3
7	1	10	0
8	11	5	1
9	33	38	0
tsA x tsJ			
1	9	1	4
2	94	40	8
3	17	18	0
4	5	16	0
5	16	3	2
6	15	13	0
7	20	12	2
8	12	11	2
9	42	5	0
tsG x tsE			
1	15	2	3
tsE x tsJ			
1	9	2	1
2	30	10	0
3	73	38	3
4	16	16	
5	37	18	0
6	10	5	1
7	20	0	0
8	30	30	5
9	24	8	0
10	9	2	0
tsG x tsI			
1	5	21	3
2	15	0	9

of not greater than 2:1. The proportion of mixed plaques in the segregants was not constant but in most cases was fairly low compared to the number of syn and syn+ plaques. These findings would be consistent with the mixed plaques being caused by the formation of temporary heterozygotes, and the morphology (mixed) indicates that after plaque initiation some segregation has already occurred.

G. Properties of segregants of mixed plaques.

To obtain further information about mixed plaque formers and to try to narrow the possible causes, an experiment was performed to distinguish between two general possibilities:- (1) The structure forming mixed plaques were completely heterozygous i. e. were not only syn/syn+ but also X/X+, Y/Y+, Z/Z+ etc. for any other pairs of markers in the cross. This type of structure would be consonant with clumps, two or more capsids or complete diploids. (2) Structures forming mixed plaques were partially heterozygous e. g. heterozygous for syn/syn+ but homozygous for other markers e. g. X, Y, Z.

This information can be obtained from a cross of the type ts+ syn x ts syn+. According to possibility (1) it would be expected that segregants from mixed plaques produced at the non-permissive temperature would be predominantly ts+ syn and ts syn+ and when the segregants are plated at 31° and 38°, 100% will plate at 31° and approximately 50% at 38°C. However from possibility (2) it might be expected that segregants would be either ts+ syn and ts+ syn+ or

ts syn and ts syn+, which when replated at 31° and 38° would give 100:100 31:38 and 100:0 31:38 respectively.

The scheme of the experiment is outlined below:-

ts+ syn x ts syn+
↓
Progeny titrated at 31°
↓
Single mixed plaques isolated and replated at 31°
↓
Segregants
↙ ↘
syn 31° 38° syn+ 31° 38°

The possible marker arrangements which could form mixed plaques are:

$$\begin{matrix} + & \text{syn} \\ X & \text{syn+} \end{matrix} \longrightarrow \begin{cases} (1) \left[\begin{matrix} + & \text{syn} \\ X & \text{syn+} \end{matrix}\right] \text{—} \begin{cases} + \text{ syn} \\ + \text{ syn} \\ X \text{ syn+} \\ X \text{ syn+} \end{cases} \\ (2a) \left[\begin{matrix} + & \text{syn} \\ & \text{syn+} \end{matrix}\right] \text{—} \begin{cases} + \text{ syn} \\ + \text{ syn+} \end{cases} \\ (2b) \left[\begin{matrix} X & \text{syn} \\ & \text{syn+} \end{matrix}\right] \text{—} \begin{cases} X \text{ syn} \\ X \text{ syn+} \end{cases} \end{cases}$$

The results of one experiment of this type are shown in Table 26. It can be seen from the results that for the crosses ts+ syn x tsIsyn+ and ts+ syn+ x tsAsyn, the great majority of the segregants from mixed plaques whether syn or syn+ behaved as ts i. e. they only grew at the permissive temperature. For the crosses ts+ syn+ x tsGsyn (1) and ts+ syn+ x tsDsyn most of the segregants both syn and syn+ behaved as wild type i. e. they grew at both the permissive and

Table 26. Behaviour of Segregants of Mixed Plaques

Cross	Plaque No. from 31°		No. of Plaques at 38°	No. of Plaques at 31°
ts+syn x tsIsyn+	1	syn	0	12
	2		0	46
	3		0	19
	4		0	167
	5		0	6
	6	syn+	28	180
	7		0	178
	8		53	280
	9		0	198
	10		0	>1000
ts+syn+x tsGsyn (1)	1	syn	0	0
	2		200	460
	3		12	15
	4		28	46
	5		71	143
	6	syn+	>1000	>1000
	7		>1000	>1000
	8		280	360
	9		21	16
	10		110	140
ts+syn+x tsGsyn (2)	1	syn	0	1
	2		0	6
	3		0	13
	4		0	14
	5		0	12
	6	syn+	120	300
	7		>1000	>1000
	8		280	>1000
	9		>1000	>1000
	10		400	640
ts+syn+x tsDsyn	1	syn	42	82
	2		0	53
	3		70	160
	4		24	120
	5		86	120
	6	syn+	>1000	>1000
	7		134	480
	8		235	640
	9		0	>1000
	10		0	>1000

Table 26 contd.

Cross	Plaque No. from 31°	No. of Plaques at 38°	No. of Plaques at 31°
ts+syn+ x tsAsyn	1 syn	0	15
	2	0	32
	3	25	36
	4	0	36
	5	0	4
	6	0	0
	7	0	1
	8	0	50
	9	0	90
	10	0	21
	11 syn+	0	>1000
	12	0	280
	13	0	5
	14	0	130
	15	0	240
	16	0	>1000
	17	0	>1000
	18	0	>1000
	19	0	>1000
	20	0	>1000
ts+syn+ x ts+syn	1 syn	57	36
	2	>1000	>1000
	3	>1000	>1000
	4	20	7
	5	87	77
	6	4	0
	7	10	3
	8	40	30
	9	2	0
	10 syn+	400	200
	11	30	28
	12	20	0
	13	320	150
	14	>1000	>1000
	15	960	400
	16	96	48
	17	6	4
	18	17	8

non-permissive temperatures. For the cross ts+ syn+ x tsGsyn (2) all the syn+ segregants behaved as wild type and all the syn segregants behaved as ts although this is possibly due to the low plaque counts at the permissive temperature. It is also possible that it is due to plaque overlap, double heterozygotes or double nucleocapsids. When the virus grew at both temperatures the amount of growth displayed at 38° was usually slightly less than the growth at 31°. From the control ts+ syn+ x ts+ syn it can be seen that this would appear to be the normal behaviour of wild type particles in such an experiment. From these preliminary experiments it would appear that the segregants from mixed plaques behave predominantly as homozygotes for unselected alleles.

H. Discussion.

From a preliminary analysis of the nature of mixed plaques it can be concluded that:-

(1) Mixed plaques are formed as a general effect of mixed infections.

(2) The proportion of mixed plaques amongst the progeny from any one cross was in most cases more than could be accounted for by the overlap of syn and syn+ plaques.

(3) From heat inactivation, sonication and U. V. inactivation experiments, the mixed plaques would appear not to be predominantly due to clumps of particles or to more than one nucleocapsid within a single envelope.

(4) There seems to be a relationship between the proportion of mixed plaques produced in a cross and the location of the mutants (with respect to the plaque morphology marker) taking part in the cross.

(5) In most cases the mixed plaques segregate into the parental types and in some cases yielded a small proportion of mixed plaques.

(6) The segregants from mixed plaques behave in most cases tested as homozygous for unselected alleles.

DISCUSSION.

This investigation has provided the first study in depth of the genome constitution and of the genetic mechanisms that characterise Herpes simplex virus Type 1. Nine temperature sensitive mutants (isolated by Professor J. H. Subak-Sharpe), have been classified into eight complementation groups. Based on a calculation from the Poisson distribution, this would suggest that the genome of HSV contains over thirty cistrons with 38° indispensable functions. (Due to the small number of mutants, this is no more than suggestive at present). It has been shown that recombination occurs in mixed infections of HSV confirming the earlier investigations of Wildy (1955). Because of the reversion of syn to syn+, three-factor crosses became possible. From each ts syn mutant ts syn+ was isolated and reciprocal three-factor crosses of the type tsXsyn x tsYsyn+ and tsXsyn+ x tsYsyn were performed. The nine ts mutants and the plaque morphology marker have been crossed in all combinations and reasonably consistent relative recombination frequencies have been obtained. The construction of a provisional linkage map has been permitted using quantitative crossing techniques.

The nine ts mutants were isolated following BUdR mutagenesis and the method of isolation avoided the possibility of any of the mutants being clonally related - a fact which gives significance to the results. Mutation using BUdR would suggest that the mutations are most likely to be transitions. The amount of back mutation

shown by each of the mutants at various times, would suggest that they are all single base change mutants. One drawback to the fact that the mutants appeared to be single step was the amount of leakiness demonstrated by certain of the mutants. The mutants tsF and tsC were the only two which were consistently leaky. Accumulation of revertants was overcome by frequent isolation and cloning of single plaque stocks but this had no effect on leakiness. However plaques due to leakiness could always be identified by their small size at the non-permissive temperature compared to the equivalent plaques at the permissive temperature.

Glasgow strain 17 was selected as the parental strain of the ts mutants as it gave the potential of introducing another marker into the system i.e. the plaque morphology marker. The original patient isolate formed syn+ (non-syncytial) plaques on BHK21/C13 cells. This spontaneously mutated to form syn (syncytial) plaques and it was stocks of syn virus which were mutagenised. The syn marker breeds true except for rare back mutation which occurs with a frequency of about 10^{-6}. When it occurs the resultant syn+ revertant is at a selective advantage and, if allowed to, builds up to form a large proportion of a stock, if it is successively passaged using large inocula for infection. Again this can be overcome by cloning from single plaque stocks. Both the markers syn and syn+ are stable at 31°, 36° and 38°C, i.e. the plaque morphology marker is independent of the temperature of incubation and it can be concluded therefore that

the gene coding for the plaque morphology marker is independent of those involved with temperature sensitivity of the mutants. The factor which causes the difference in plaque morphology is not known. The syn variant forms syncytial plaques caused by the fusion of one infected cell with the surrounding non-infected cells to form giant multinucleate fused cells. It is assumed that the virus codes either directly or indirectly for a protein which causes fusion of cell membranes and hence allows passage of virus from cell to cell without release into the extracellular fluid. Whether fusion is a prerequisite of virus mobility or the result of cell to cell passage is a matter of conjecture. The syn+ variant presumably lacks or is altered in the gene which causes cell fusion and hence virus infection results in the death of the cell with the release of virus capable of infecting surrounding cells. The type of lesion caused in vivo with HSV Type 1 would suggest that syn may be the true wild type, as the lesions appear to be caused by cell to cell spread of virus without extracellular release.

The one-step growth curves of Glasgow strain 17 and its ts mutants show similar patterns to those observed with other strains of HSV Type 1. The wild type virus grows equally well at all three temperatures i.e. 31°, 36° and 38°C whereas the ts mutants show a considerably reduced rate of growth at 36° and 38°. At 31° the ts mutants appear to replicate more slowly than the wild type virus. This observation is similar to that observed with HSV Type 2 where it has also been shown that the ts mutants replicate more slowly

than the wild type virus at the permissive temperature (Halliburton, 1972). At present this is an unexplained phenomenon which warrants further study. The growth patterns observed with the Type 1 mutants are in good agreement with the e.o.p. tests. The curves show marked similarity to those observed with the wild type virus and ts mutants of Frog virus 3 (FV/3) (Naegele and Granoff, 1971). The growth patterns of six BUdR isolated ts mutants of FV/3 were compared to those of the wild type virus and it was shown that the wild type virus grew equally well at both the permissive and non-permissive temperatures - the first infectious virus appearing at 4 hrs and increasing to 24 hrs post infection. The ts mutants on the other hand were shown to replicate more slowly than the wild type but the final yields were essentially the same. The yields of the mutants at the non-permissive temperature ranged from 0.4-3.6% of the yields produced at the permissive temperature.

The heat stability of the ts mutants was examined to ascertain whether the temperature sensitivity of these mutants arose from an alteration in a structural protein associated with virus infectivity. Experiments were carried out at two temperatures 45° and 55°. The results indicated that at these two temperatures, the ts mutants were neither more heat stable nor heat labile than the wild type virus. The data suggested therefore that if one of the ts mutants contained an altered structural protein it was not manifested by increased heat sensitivity. Heat inactivation studies with ts mutants of Vaccinia

virus (Basilico and Joklik, 1968), showed that of two ts mutants which gave similar growth patterns, one showed 0.1% survival after 90 mins at 54° and the other paralleled the wild type in giving 10% survival under identical conditions. Experiments with FV/3 showed that the inactivation rates (1% survival after 6 mins) of 11 ts mutants and the parent strain at 52°C were the same (Naegele and Granoff, 1971). Takemoto and Martin (1970), working with a large plaque variant of SV40 (SV L) which was shown to be temperature sensitive, compared its thermolability at 50°C with that of (SV-S), a small plaque variant which was not temperature sensitive. The SV-S strain was shown to be completely stable after two hours at 50°, whereas the SV-L strain gave 0.001% survival under identical conditions. Working with Sindbis virus (Pfefferkorn and Burge, 1967) showed that of several ts mutants tested for thermolability, those which were RNA-ve were slightly less heat stable than the wild type and the majority of the RNA +ve mutants were more heat labile than the RNA -ve mutants. Although our HSV ts mutants have been divided into those which make DNA and those which do not (Mechie et al., 1972), this division has not been shown to carry over into their thermosensitivity. Perhaps the most extensive work on thermosensitivity of ts mutants of animal viruses is that of McCahon and Cooper (1969) on Polio virus. They showed that 10 out of 29 ts mutants of Polio virus lost infectivity more rapidly at 45°C than did the wild type virus and they therefore concluded that these 10 mutants had structural

protein defects. In addition, this division into structural and non-structural protein defectives accorded very closely with their position on the genetic map enabling the region of the genetic map specifying structural proteins to be defined. It is hoped that with the isolation of more ts mutants of HSV that some will be found which are more heat sensitive than the wild type and it may well be possible to parallel the work with Polio virus and assign those concerned with structural proteins to one specific region of the genetic map. The one obvious drawback to fulfilling this proposal is the size of the genome of HSV (100×10^6 daltons) compared to that of Polio virus (2.2×10^6 daltons). The DNA of HSV could therefore code for about 100 averaged sized proteins whereas the RNA of polio virus has the coding capacity for about 10 such proteins.

The results of complementation tests suggested that the temperature sensitive defects in most of the mutants, with the exception of tsB and tsB2, affected different cistrons. This conclusion that the mutants identified different genes was generally supported by the recombination data.

Two types of complementation test were used to assign mutations to specific genes. The results of the more standard yield test correlated reasonably well with those of the infectious centre assay. It was felt that the use of the two types of test gave weight to our conclusions. It is true that recombinants arising in a mixedly infected cell could contribute to the formation of an infectious centre

plaque at the non-permissive temperature. However the test is quite unambiguous unless recombination in the absence of complementation occurred with comparable frequency. We have shown that the part played by recombination in complementation tests is on average very small and in most cases there was no evidence of recombination taking place at all. It is suggested that where there was evidence of wild type particles occurring in the yield from mixed infections at the non-permissive temperature, that the level was greatly influenced by the background growth (38°) of the individual mutants. When more mutants in the same cistron are available the part played by recombination will be more critically testable. The only test that has so far been possible (between tsB and tsB2) has been entirely negative. The infectious centre test has proven itself to be a reasonably reliable method and in fact without this method, complementation assignments in HSV Type 2 could not have been made (Timbury, 1972). Some variability in complementation indices from different experiments with the same pair of mutants has been observed. Owing to the fact that we have had two types of test available, this has not led to problems in classification and has not altered or affected our conclusions about the assignments of mutants to cistrons.

The complementation indices were lower than those obtained with some viruses e. g. VSV (Pringle, 1970; 1971) and Adeno virus 5 (Williams, 1971) but were higher than those reported with most

other viruses. The efficiency of complementation was about 20% which is higher than that obtained with most other viruses. Comparing our results in detail with those of complementation tests involving other viruses:- (a) The degree of complementation found between a large number of ts mutants of Polio virus was very small. One pair of mutants was studied in some detail and although it was shown that complementation occurred up to 14 times the background leak rate, the efficiency was found to be 0.1% for the ts+ yields. The complementation test therefore proved to be of no value in demonstrating cistron groups in Polio virus (Cooper, 1965). (b) With Sindbis virus the yield from mixed infections at the non-permissive temperature exceeded the yields from the two parents grown separately, by a factor of 3-300, although the efficiency was quite low at 1-3% of the yield of the parental virus at the permissive temperature. The efficiency was found not to be affected by the multiplicity of infection within the limits of 2-20 p.f.u./cell (Burge and Pfefferkorn, 1966; 1968). With HSV Type 1 we have found that the level of complementation is not changed by a 2-fold increase in total m.o.i. (5-10 p.f.u./cell). (c) In mixed infections of ts mutants of Influenza virus at the restrictive temperature, the yield was about 30-fold over that of the single parent controls, although 25% of the 30-fold increase was found to be wild type (Simpson and Hirst, 1968). (d) Complementation yield experiments involving six ts mutants of Group 1 and six of Group 4 of VSV gave complementation indices ranging from 28-912. The

efficiency of complementation with VSV was found in general to be fairly high (Pringle, 1970). (e) With Rabbitpox virus, complementation tests involving 18 ts mutants, gave yield increases of 5-10 fold over that of single parent infections (Padgett and Tomkins, 1968). (f) Pairwise crosses involving 6 ts mutants of FV/3 gave complementation indices ranging from 9-126 (Naegele and Granoff, 1971). (g) With Adeno virus 5, the yields from mixed infections at the non-permissive temperature ranged from a six fold increase over single parent infections to a 1.1×10^6 fold increase (Williams and Ustacelebi, 1971).

It would appear therefore that there are considerable differences in the levels and efficiency of complementation with different virus groups and also within groups. There does not seem to be any correlation between efficiency and the nucleic acid type of the virus i.e. DNA viruses do not appear to complement more efficiently than RNA viruses or vice versa. It also does not seem to be controlled by the size of the genetic material. Complementation indices obtained with adenovirus 5 whose DNA has a molecular weight of 23×10^6 daltons, are higher than those obtained with HSV Type 1 with a DNA molecular weight of 105×10^6 daltons. Within the Herpes group the complementation indices obtained with HSV Type 2 mutants are lower than those obtained with Type 1. In fact complementation is not demonstrable with Type 2 virus using the standard yield experiment but only becomes apparent in an infectious centre assay (Timbury, 1971). It is possible that at least for viruses multiplying in the cell

nucleus that the average yield of virus (burst size) may play a considerable part in the levels of complementation obtained. This is borne out by the average yields of VSV and Adenovirus 5 which are about 10-100 times higher than for HSV Type 1 (100 p.f.u. /cell). Substance is also given to this theory by the fact that HSV Type 2 has a lower average yield (10 p.f.u. /cell) than HSV Type 1.

With complementation tests of HSV Type 1 the only problem which has arisen has been the variability observed from one set of experiments to another. (This has also been observed in the recombination studies and will be discussed in that section).

There are still some experiments which if carried out would give a more comprehensive picture of the process of complementation between ts mutants of HSV Type 1. It would be interesting to study further:- (1) The reasons for complementation between two mutants occurring at 36° but not at 38°, as was observed in the cross tsA x tsJ and on certain occasions between tsA x tsE. (2) The effect of m.o.i. on complementation indices especially between DNA +ve and DNA -ve mutants. Work on Sindbis virus has shown that altering the m.o.i. with two RNA +ve mutants had little effect on the efficiency of complementation but when one parent was RNA -ve, increasing the m.o.i. of both parents was found to increase the efficiency of complementation (Burge and Pfefferkorn, 1968). (3) The genotypes and phenotypes produced in complementation yields. It is known that in most cases no wild type recombinants are produced

i. e. the yield is 100% mutant but it is not known whether the yield contains both mutants and if both are produced whether they are in equal proportions. Infectious centre assays with one ts mutant syn and the other syn+ have shown that both genotypes are functioning within the same infected cell in that infectious centre plaques are of a mixed morphology, i. e. partially syncytial and partially non-syncytial. To confirm this in yield experiments it would be necessary to do (a) back crosses with each of the two parental viruses or (b) to look at the DNA phenotype of the progeny virus when mixed infections were between DNA +ve and DNA -ve mutants or (c) complementation tests with the parental strains. (4) The kinetics of complementation in terms of the time of release of progeny from a mixed infection at the non-permissive temperature. This has been done with ts+ progeny from a mixed infection at the permissive temperature and was found to parallel the time course of growth of single mutants. There is no reason to suppose that the release of virus at the non-permissive temperature should follow any different kinetics, if both mutants are complementing. (5) To improve complementation indices by obtaining good stocks with low backgrounds at the non-permissive temperature. Perhaps more important than any of the above, would be the establishment of a spot test to ascertain qualitatively whether or not complementation is taking place between two mutants. This will become increasingly important with the isolation of new mutants. The possibility of

developing such a test seems feasible.

A provisional linkage map of HSV Type 1 has been constructed using quantitative crossing techniques. The fact that the great majority of putative ts+ recombinants bred true on progeny testing and were therefore true recombinants justified our quantitative measurement of recombination. The recombination frequencies were calculated as twice the frequency of the ts+ recombinant class on the assumption that the tsXtsY reciprocal recombinant is produced with equal frequency. Results in crosses of the type tsXsyn+ x ts+syn have shown that all four expected genotypes are present in the resulting progeny but we have not attempted to identify double recombinants in the progeny from tsX x tsY crosses. The results of several series of three-factor crosses have shown that although infection is with a theoretical average of 5 p.f.u./cell of both tsX and tsY, many individual crosses did not yield equal frequencies of syn and syn+ markers in the progeny when assayed at the permissive temperature. This could be due to the fact that:- (1) one mutant genome suppresses the replication of the other or (2) one mutant's genome outgrows the other or (3) the input 5 p.f.u./cell, while representing the multiplicity of exposure to infection by fully infectious particles does not measure the number of genomes or genes copies which enter each cell and participate in the subsequent genetic events. The number could well be considerably higher and moreover vary with the mutant stock used and its age.

Possibility one is ruled out by the fact that at different times either syn or syn+ have been found to be in excess in the progeny from the same cross. Possibility two also seems to be unlikely as the growth patterns of most of the mutants at the permissive temperatures are similar. The exceptions to this are the mutants tsA and tsJ which are known to grow more slowly than the other mutants. However it is by no means only or consistently crosses involving these two mutants which show the syn, syn+ inequality in the progeny virus. We would suggest that the third possibility is the most likely explanation since it is known that the E. M. particle/p. f. u. ratios in Herpes stocks can range widely. In our laboratory very good stocks with ratios of 5 or less are obtained, but poorer stocks with ratios of 20 or even occasionally 100 are also obtained. It is not known how this ratio influences the recombination process, but if some non-infectious particles can participate in events leading to recombinants then unequal input of non infectious particles but equal input of infectious particles would result in unequal ratios of markers in the infectious progeny. Obviously this could affect the observed recombination frequencies and we would suggest that variation in recombination frequencies and in complementation indices from one set of experiments to another could well be influenced by the particle:p. f. u. ratio of the stocks being used and the proportion of non-infectious particles taking part in the process. It is not suggested that this is the only factor which causes variation. It is obvious that topography

and the physiological state of the cells at any one time plays a considerable part in causing variation.

For the present therefore the only in-built control in the recombination system is to monitor the ratio of syn:syn+ in the total progeny at 31°. If the parental markers are found in a 1:1 ratio then this is probably a good indication that the number of partners in the mating pool were about equal. On the assumption that the mating kinetics of HSV Type 1 are approximated by the Visconti-Delbrück[11] theory it is to be expected that only those crosses having a near 1:1 marker ratio in the progeny would be comparable in terms of relative recombination frequencies. Therefore until the gene input into crosses is better understood it was decided to discard for mapping purposes those crosses in any series in which the ratio of syn:syn+ was greater than 3:1 or 1:3 at the permissive temperature. This selection of results appears to have eliminated many ambiguities.

The data from one complete set of reciprocal three-factor crosses was used to order the markers. The order was reasonably consistent inspite of variation in the recombination frequencies for a given pair of markers in separate repeat experiments and the data from a series of two factor cross experiments fitted well with the proposed order. The map which spans some 25-30 map units can be expected to extend with the isolation of further mutants and the identification of new genes.

The plaque morphology marker has proved most useful in two

respects in that it has allowed the monitoring of output ratios from mixed infections and it has greatly facilitated the mapping of the genome by permitting three-factor crosses to be made. By comparing the frequency of syn and syn+ plaques among the selected class of ts+ recombinants, the order of the two ts markers in terms of their proximity to the syn gene is obtained. Taken together with the calculated recombination frequencies this facilitates the ordering of the mutant sites and gives confidence in the map particularly where the quantitation of the map distances is subject to some variability.

With the available markers the proposed map provides the best fit of the data obtained. There is no evidence that the genetic map of HSV is other than linear. The linkage distances and the map order are provisional, but while it is expected that minor modifications may have to be made in the future, it is not anticipated that further experiments will radically alter the general features. The alternative positions of the plaque morphology marker on either side of tsJ is accounted for by the fact that since only one selected class of recombinant is scored, the three-factor cross only permits the determination of which ts mutant is most proximal to syn but not on which side of the mutant syn is located. In comparison with other animal viruses, recombination has been demonstrated in both RNA and DNA viruses, but only with Influenza virus and Polio virus has an attempt been made to construct genetic maps. With Influenza virus, a linkage map has been constructed from two factor cross data

involving ts mutants (Mackenzie, 1970). The recombination frequencies were found not to increase during the growth cycle and it was presumed that a single round of mating took place early in the growth cycle. However this conclusion did not fit with the high R. F. values obtained and so it was suggested that multiple rounds of mating might take place, followed by prompt encapsidation of the RNA. High recombination frequencies in Influenza virus had been encountered previously by Simpson and Hirst (1968) and they postulated that the high values may be due to the occurrence of subgenomic fragments of RNA in the infected cell. Simpson and Hirst suggested that at the time of virus maturation these fragments might be exchanged and assembled in a random fashion and therefore the probability would be high that any given virion would contain genetic material from more than one parent. Their postulation was substantiated by the finding that the RNA of the virus strain used in the recombination studies could be resolved into 5 or 6 pieces by acrylamide gel electrophoresis (Pons and Hirst, 1968). At face value the argument for genome pieces accounting for the high R. F. values seems more sound.

With HSV Type 1 we have found in several crosses in which the production of ts+ recombinants has been followed with time, that recombination frequencies increased with time and followed fairly closely the time course of growth of the parent viruses involved in the cross. It would appear therefore that we are dealing with multiple rounds of mating, possibly involving progeny strands.

Undoubtedly the most comprehensive genetic analysis of any animal virus is that of Polio virus. By stringently controlling the system, Cooper (1968) obtained an additive linear genetic map of Polio virus ts mutants that comprised one linkage group. The recombination frequencies were found to be reproducible and characteristic of each pair of mutants. Three factor crosses were facilitated by the use of a ts mutant in a guanidine resistant form. Like our plaque morphology marker it was advantageous that the 'g' marker was found to be located about the middle of the map. Maps involving other markers than ts have also been obtained for Polio virus (Bengtsson, 1968; Hirst, 1962). It was shown that mating events were not obligatorily linked with replicative events and it was thought that multiple rounds of mating did not occur to any great extent. It is thought that with Polio virus double stranded RNA is a prerequisite of mating events. As with HSV the doubly defective ts recombinants were not looked for but the self consistent nature of the map suggested that recombination was reciprocal.

Our recombination studies with HSV have by no means come to an end. It is hoped in the future to:-

(1) Extend the map following the isolation of new mutants and if possible to dispense with those mutants which at present give rise to problems of interpretation of results due to their leakiness.

(2) Isolate the doubly defective ts mutants and determine if it

is justified in supposing that the double ts mutant is produced with the same frequency as the ts+ recombinant.

(3) In conjunction with (2) to identify the four types produced in all crosses of the type ts+ syn x ts syn+ and the reciprocal and determine the proportions of each class produced.

(4) Determine the part played by non-infectious particles in recombination processes by controlling the particle:p. f. u. ratios of virus stocks taking part in a cross and if possible to make some estimate of the amount of non-infectious particles taking part in a cross.

(5) Determine the effect of differences in total m. o. i. on recombination frequencies and of varying the 1:1 ratio of the two parents taking part in a cross.

(6) Monitor the time course of production of ts+ recombinants for all possible crosses.

(7) Carry out single burst experiments i. e. by examining the progeny from single cell infections to determine something more about the processes of recombination and to see if events in a single cell are comparable with those observed in a cell population.

During the recombination studies it became apparent that the progeny virus, as well as containing syn and syn+ plaques, contained plaques of a mixed morphology i. e. partially syncytial and partially non-syncytial. It has been shown that the proportion of mixed plaques

obtained in the progeny from any one cross is more than could be accounted for by the overlap of two individual syn and syn+ plaques. In control experiments plating mixtures of tsXsyn x tsXsyn+ stocks, the proportion of overlapping plaques increases with increasing plaque numbers. In mixed infections of the type tsXsyn x tsYsyn+ the proportion of mixed plaques in the progeny was not related to the total number of plaques. On further investigation it was shown that the mixed plaques represent structures which segregate upon replication to produce primarily true breeding progeny i.e. syn and syn+, although a small proportion do again yield mixed plaques. Mixed plaques could originate from clumps of virus particles, or from more than one complete nucleocapsid being present within a single envelope. Sonication and heat inactivation experiments showed that the fall off in mixed plaques after sonication or heat treatment was not significantly different from the inactivation patterns obtained with total virus and therefore it concluded that the mixed plaques are unlikely to be caused by clumps of particles, although the fact that clumps of particles could contribute to a very minor extent to the formation of mixed plaques is not completely ruled out. Ultra-violet light inactivation studies showed that the inactivation curves of mixed plaques were not multiple hit curves, as would be expected if the mixed plaques were due to more than one nucleocapsid within the same envelope. Their rate of fall off with time paralleled that of the total population. Recent observations have suggested

that the frequency of mixed plaques is a function of the actual markers used in the cross. Crosses involving markers around the region of tsJ yield a higher proportion of mixed plaques amongst the ts+ progeny than do crosses involving markers near the extremities of the genetic map. This constitutes suggestive evidence that their formation is related to the events which lead to the production of recombinants and it is suggested that on the evidence to date that mixed plaques may be due to some form of partially heterozygous particle.

The occurrence of heterozygotes and in fact mixed plaque heterozygotes was first recognised in the bacteriophage T2 by Hershey and Chase (1951). The general features of heterozygotes described by Hershey and Chase and later workers were as follows:-

(1) They were not restricted to any particular pair of alleles but were found to arise with the same frequency, about 2% of the progeny, with respect to every locus examined.

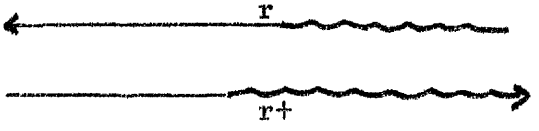
(2) Two types of heterozygote are possible (a) heterozygous for more than one locus or (b) heterozygous for one locus and homozygous for others.

(3) By scoring the frequency of occurrence of double heterozygotes as a function of the distance between two markers, the average length of the heterozygous region was estimated to be approximately 1% of the DNA molecule length.

(4) When crosses are made between viruses which differ by three

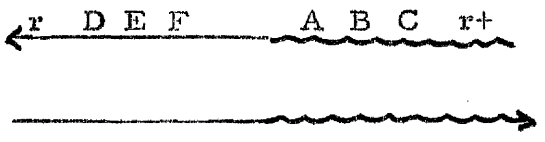
markers, particles heterozygous for the middle marker appear to be recombinant for the outside markers.

(5) The heterozygotes represented intermediate structures in the recombination process. They are being formed as a normal stage in the production of recombinants; they are continuously being made in the mating pool and are lost at the same rate during segregation. What of the physical nature of the heterozygous region? With the T-even phages two models have been proposed to explain the formation of heterozygous particles. The first of these is a heteroduplex model, represented by a covalently bonded DNA molecule containing a limited heterozygous region in which each of the two complementary polynucleotide chains carries one of the two parental alleles.



The heterozygote would evidently segregate into homozygous r and r+ recombinants upon semiconservative replication.

The second model which satisfies the requirement is a terminally redundant heterozygote.



During replication in the infected cell, genetic recombination can proceed within the region of terminal redundancy of two daughter

molecules, giving rise to molecular concatomers. These then have to be cut into virus genome sized pieces. If the cutting process starts to take its measure of genome length always from the same site, then only one kind of terminally redundant virus genome arises. Hence terminally redundant heterozygotes arise only for genes residing in that sector. If however the cutting process occurs randomly then there would arise a collection of virus genomes whose terminal redundancy would be circularly permuted and hence redundancy heterozygotes could arise for any one gene. The latter case is true for the T even phages.

The heterozygotes were found to contain normal double stranded DNA and it would not be surprising if similar events occurred in animal viruses containing double stranded DNA. The HSV mixed plaques do appear to have some properties in common with phage heterozygotes:-

(1) They are produced in crosses of the type syn x syn+, irrespective of the other markers taking part in the cross.

(2) The mixed plaques segregate into syn and syn+ plaques with in some cases a small proportion of the plaques remaining mixed.

(3) From preliminary experiments, it would appear that the viral genome giving rise to mixed plaques are homozygous for outside markers.

(4) The frequency of the mixed plaques produced in a cross

syn x syn+ appears to depend on the ts markers taking part in the cross. (This is not true for the T even phages but is true for T1).

This investigation into the nature of mixed plaques is very much in its infancy. We feel fairly certain that for the most part they are not due to clumps of particles or structures of that nature. This will be more readily testable when a method of artificially clumping HSV particles has been developed. In this way the sonication, heat inactivation and U. V. inactivation results will become more meaningful. From these preliminary investigations we would suggest that mixed plaques may be due to structures intermediate in the recombination process. When more investigations of a genetic nature have been carried out, it is hoped to propose a model for the heterozygous region.

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